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PRINCIPAL INVESTIGATOR: Jerry D. Hendricks, Ph.D.

CONTRACTING ORGANIZATION: Oregon State University
Corvallis, Oregon 97331-3804

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FOREWORD

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Jerry D. Hendrick 8/27/96
#1 - Signature Date

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INTRODUCTION

The traditional rodent bioassays for detection of suspect chemical carcinogens are so expensive (up to \$1 million per chemical) and slow (at least 2 years) that only a few hundred of the many thousands of industrial chemicals have been tested. The possibilities for markedly increased testing are minimal, and the potential for researching complex environmental mixtures is even more discouraging. Although short-term screening assays such as the Ames bacterial mutagen assay (McCann and Ames, 1977) are mechanistically informative, they do not produce cancer as the endpoint, nor can any *in vitro* test match the fundamentally important physiological and pharmacokinetic parameters which often determine if a chemical will cause cancer in whole animals. As a result, there is a continuing need to develop alternative vertebrate models to supplement the use of rodent models for identifying and understanding the action of agents that cause cancer. Species that may be useful as both laboratory models and field monitors are of special interest.

Fish have received increased attention as useful alternative cancer models. Fish are especially appropriate species for testing and monitoring carcinogenic chemicals in our surface and ground waters, which are susceptible to contamination by toxic chemicals from industrial dump sites, munitions manufacturing and testing sites, fuel depots, and other activities that deposit potentially hazardous chemicals into the environment. Over the past three decades, our laboratory has developed the rainbow trout as the most widely recognized fish model for carcinogenesis research (Bailey *et al.*, 1996). Many desirable attributes of this model have been established, including high sensitivity, low cost, a relatively large data base, well characterized histopathology, established husbandry requirements, a body size range from milligrams to kilograms, and a useful non-mammalian comparative status.

While trout will continue to be highly useful, they have limited use as a field monitor, and have certain other disadvantages that may be offset by other fish with different biological features. For example, genetic studies in trout are severely restricted by a 2-3 year maturation period, feeding costs rise with age, tumor studies require at least 9 months, and can only be initiated twice per year at most using photoperiod control of spawning. By comparison, many aquarium fish reach maturity at 2-3 months, spawn on a continuous basis, develop tumors somewhat more rapidly, and have lower feed costs. The Japanese medaka (*Oryzias latipes*) is being widely developed as one such model. It is responsive to several carcinogens (Ishikawa *et al.*, 1975; Aoki and Matsudaira, 1977; Hawkins *et al.*, 1988b), adapts to a wide range of water conditions, and is economical with regard to space and rearing costs. However, as we have observed with trout, no single species will respond to every insult delivered. Thus, in the development of fish models as sentinels to detect waterborne carcinogenic hazards *in situ*, reliance on a single species may yield incorrect information of a false positive or false negative nature. Ideally, a second aquarium species would complement the medaka by responding to a wide range of carcinogens and environmental modulators, and would provide its own unique strengths to enhance our overall goal of detecting carcinogen hazards and understanding the mechanisms underlying the observed results. In the final analysis it is mechanistic information, rather than dose-response data, that can be most readily extrapolated to predict human risk.

Among the several aquarium species being developed (i.e. guppy, platyfish, *Poeciliopsis*, *Rivulus*), we believed the zebrafish (*Brachydanio rerio*), a small cyprinid fish, held exceptional promise. Of greatest interest and potential utility was the unique and superb history of genetics and developmental biology that has been gathered for zebrafish (Laale, 1977). Completely homozygous cloned lines have been developed (Streisinger *et al.*, 1981) that could be of great use in cancer research. The embryos are transparent and offer a superb model for easily visualizing the effects of environmental toxins on embryonic development (Hanneman *et al.*, 1988). Embryos are so small that direct microinjection may permit testing of rare suspect compounds at the *picogram* level.

In 1965, zebrafish were the first aquarium species in which chemically initiated tumors were demonstrated (Stanton, 1965), but for some reason, the species has received little further attention for carcinogenesis research since that time. The aim of this overall project was to greatly expand the data base of carcinogen classes tested in the zebrafish, to fully characterize neoplastic responses in this species, and to understand the responses in terms of procarcinogen metabolism, DNA adduction and repair. In addition, we wanted to determine the role of oncogenes in the carcinogenic response and attempt to introduce transgenes into zebrafish that would make them even more responsive to carcinogens. Some tandem studies with the medaka were conducted to provide a basis for comparison between the two species.

BODY

CARCINOGENESIS STUDIES (Objectives 1, 2 and 3)

METHODS

Chemicals

We chose to work with six carcinogens and obtained them from the following sources: N-nitrosodiethylamine (DEN) was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). N-nitrosodimethylamine (DMN), aflatoxin B₁ (AFB₁), methyl-azoxymethanol acetate (MAM-Ac), and dehydroepiandrosterone (DHEA) were purchased from Sigma Chemical Co. (St. Louis, MO). N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz[a]anthracene (DMBA) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Animals

Zebrafish were initially obtained from 5-D Tropical Fish (Plant City, FL). Thereafter, they were spawned and reared at the Food Toxicology and Nutrition Laboratory (FTNL), Oregon State University. Japanese medaka (*Oryzias latipes*) were obtained from Carolina Biological Supply Co. (Burlington, NC).

Culture Conditions

Fish were reared in the well water normally used for salmonid culture at the FTNL, but this water had to be treated in the following ways. It was run through a column of plastic coils to remove excess nitrogen gas, buffered to a pH of 7.0-7.2 with a phosphate buffering system, and heated to a temperature between 24 and 26°C. Experimental groups of fish were held in 110 L tanks. Ten times weekly, 4 L of new water was added to these tanks and 4 L was removed through an overflow system. A 14-hr light/10-hr dark photoperiod cycle was used and each tank was equipped with an airstone for aeration.

Fish were fed according to the following schedule. About five days after hatching, zebrafish larvae were fed Microfeast Plus larval diet (Provesta Corp., Bartlesville, OK) 3-5 times daily. After about two wk, they were started on brine shrimp, and slowly (over 3-5 days) weaned off Microfeast. They received brine shrimp only until about 6 wk old, and from that time on they were fed Oregon Test Diet (OTD) (Lee *et al.*, 1991) twice daily and brine shrimp once daily. This diet regimen was continued throughout the experiments except in the dietary exposure groups, described below.

Diets

Purified casein diet (PC diet), which was developed at the University of California, Davis, for use with Japanese medaka (DeKoven *et al.*, 1992), was used as the basic diet in

our feeding experiments. However, for dietary carcinogen exposures, we preferred to have the diet in a gelatinized rather than powdered form. Thus we added 2% gelatin to the basic PC diet formulation, and made a moist diet by thoroughly mixing 65% hot water ($\approx 55^{\circ}\text{C}$) with 35% dry mix and cooling in a refrigerator. In this form the diet retained its integrity in the water longer, resulting in more complete consumption of the administered amount.

Fish used in the fry and embryo exposure experiments were fed OTD, which has been developed at OSU for use with rainbow trout and brine shrimp.

Dietary Exposure

DEN or DMN, at doses of 500, 1,000, or 2,000 mg/kg (ppm) diet was added to the hot water before combining with the other ingredients of the modified PC (MPC) diet, and mixing as described above. The diets were fed by expressing the diet through a fine meshed screen mounted on a caulking gun and cutting with a spatula.

Duplicate groups of 100, 8-wk-old zebrafish, selected randomly with regard to sex, were placed in 110 L tanks and fed the MPC diet or MPC diet plus 500, 1,000, or 2,000 ppm DEN or DMN for 12 wk. Fish were then maintained on the MPC diet for an additional 14 wk until the experiment was terminated.

For AFB₁, duplicate groups of 90, 8-wk-old zebrafish were fed the MPC diet or MPC diet plus 10, 20, or 30 ppm AFB₁ for 3 mo. At that time, one of the duplicate groups from each treatment was started on MPC diet containing 444 ppm DHEA and fed this diet for an additional 6 mo. The other group from each treatment received MPC diet only. All fish were killed and fixed in Bouin's solution at the end of the combined 9 mo.

Dietary exposure of zebrafish to MAM-Ac was accomplished in similar fashion. Duplicate groups of 90, 8-wk-old zebrafish were fed MPC diet or MPC diet plus 500, 1000 or 2000 ppm MAM-Ac for 12 wk, maintained on MPC diet for another 12 wk, and terminated to determine neoplastic response. Single groups of 100 medaka (8 wk old) were fed the MPC diet containing 1000 or 2000 ppm doses of MAM-Ac for 12 wk and handled the same way as the zebrafish for comparative purposes.

Duplicate groups of 100, 8-wk-old zebrafish were fed MPC diet or MPC diet plus 500, 1000 or 2000 ppm MNNG for 12 wk. They were then placed back on MPC diet for an additional 12 wk and terminated.

For DMBA, duplicate groups of 80, 8-wk-old zebrafish were exposed to MPC diet or MPC diet plus 100, 500 or 1000 ppm DMBA for 4 mo. At that time, they were all placed on MPC diet, fed for an additional 3 mo and terminated.

Fry Bath Exposure

Single groups of 120 zebrafish fry, 16 days post-hatch, were exposed to buffered water solutions of DEN (no solvent carrier was used) at concentrations of 500, 1,000, 1,500,

and 2,000 ppm for 24 hr under static conditions. Control fish were held under similar conditions but without the DEN. After exposure, each group of fish was placed in a 110 L glass tank and fed OTD and brine shrimp for 12 mo. At termination, all fish were killed and fixed for histopathological diagnosis. The procedures for DMN exposure were the same, but the DMN doses used were 250, 500, 1,000, 2,000, and 2,500 ppm. Abnormally high mortalities occurred from time to time during the experiment, resulting from a parasitic outbreak of *Oodinium sp.* Acriflavine, at a dose of 5.4 ppm, was added to the water as needed to control this problem.

Duplicate groups of 100 zebrafish fry, 21 days post-hatch, were exposed to aqueous solutions of AFB₁ at 0.5 and 1.0 ppm for 24 hr. Nine months later, no neoplasms were observed in these fish. These negative results are not included in the Results section.

Single groups of 100 zebrafish fry, 21 days post-hatch, were exposed to MAM-Ac, dissolved directly in buffered water at concentrations of 6.25, 12.5, 25, 50, 75 and 100 ppm, under static conditions for 2 hr. Control fish were handled the same way without exposure to MAM-Ac. After exposure the fish were placed in 110 L tanks, fed OTD and brine shrimp for 12 mo and terminated as previously described.

Single groups of 100 zebrafish fry, 21 days post-hatch, were exposed to MNNG at concentrations of 0.5, 1.0, 1.5 and 2.0 ppm, directly dissolved in buffered water under static conditions for 24 hr. Control fish from the same stocks were maintained under identical holding conditions without exposure to MNNG. Doses above 0.5 ppm resulted in 100 percent mortality. Therefore, only the control and 0.5 ppm groups were reared on OTD and brine shrimp until sacrifice 12 mo later.

Single groups of 100, 21-day-old zebrafish fry were exposed to static buffered water solutions of DMBA at concentrations of 0, 1.25, 2.5 and 5.0 ppm. DMSO at 1% was used as a carrier for DMBA. Fish were fed OTD and brine shrimp for 9 mo and terminated to determine neoplastic response.

Embryo Bath Exposure

Duplicate groups of 150 zebrafish embryos, 60 hr post-fertilization, were placed in static, buffered water solutions of DEN at 1,000, 2,000, and 3,000 ppm for 24 hr. After treatment the embryos were rinsed in clean water and placed in 1.5 L beakers until hatching at 96 hr. The resulting fry were started on feed and kept in the 1.5 L beakers for one mo before transferring to 110 L glass tanks. They were fed OTD and brine shrimp for 12 mo, killed in tricaine methane-sulfonate (MS 222) anesthetic and fixed for histopathological diagnosis. No embryos were exposed to DMN.

Duplicate groups of 150 zebrafish embryos, 83 hr post-fertilization, were exposed to AFB₁ at doses of 0, 0.25, 0.5 and 1.0 ppm for 1 hr. AFB₁ was dissolved in buffered water containing 1 ml 95% ethanol as a carrier; control groups also included the ethanol carrier. After treatment the embryos were rinsed in clean water and placed in 1.5 L beakers until hatching at 96 hr. The resulting fry were started on feed and kept in the 1.5 L beakers for

one mo before transferring to 110 L glass tanks. They were fed OTD and brine shrimp for 10 mo, killed in tricaine methane-sulfonate (MS 222) anesthetic and fixed for histopathological diagnosis.

Duplicate groups of 150 zebrafish embryos, 72 hr post-fertilization, were exposed to static buffered water solutions of 0, 10, 25 and 50 ppm MAM-Ac for 12 hr. After exposure they were transferred to clean, buffered water in 1.5 L beakers where they were allowed to hatch and begin feeding. Finally, they were placed in 110 L tanks, fed OTD and brine shrimp for 12 mo and terminated to determine neoplastic response.

Duplicate groups of 150 zebrafish embryos, 83 hr post-fertilization, were incubated in buffered aqueous solutions of 1, 5 and 10 ppm MNNG for 1 hr. After exposure they were transferred to clean, buffered water in 1.5 L beakers where they were allowed to hatch and begin feeding. Finally, they were placed in 110 L tanks, fed OTD and brine shrimp for 12 mo and terminated to determine neoplastic response.

Duplicate groups of 150 zebrafish embryos, 60 hr post-fertilization, were incubated in buffered aqueous solutions of 0.25, 0.5 and 1.0 ppm DMBA for 24 hr. After exposure they were transferred to clean, buffered water in 1.5 L beakers where they were allowed to hatch and begin feeding. Finally, they were placed in 110 L tanks, fed OTD and brine shrimp for 12 mo and terminated to determine neoplastic response.

Embryo Microinjection

A single group of 100 zebrafish embryos, 72 hr post-fertilization, was microinjected with 96 ng of MNNG per embryo. The MNNG was dissolved in a combination of ethanol, DMSO and water. A group of 40 zebrafish embryos of the same age were also microinjected with the ethanol/DMSO/water carrier. After exposure all embryos were transferred to clean, buffered water in 1.5 L beakers where they were allowed to hatch and begin feeding. Finally, they were placed in 110 L tanks, fed OTD and brine shrimp for 12 mo and terminated to determine neoplastic response.

A single group of 100 zebrafish embryos, 72 hr post-fertilization, was microinjected with 0.376 ng of AFB₁ per embryo. The carrier for the AFB₁ was 95% ethanol. Forty similar aged embryos were microinjected with the ethanol carrier. After exposure all embryos were transferred to clean, buffered water in 1.5 L beakers where they were allowed to hatch and begin feeding. Finally, they were placed in 110 L tanks and fed OTD and brine shrimp. This experiment is still in progress, 10 mo post-treatment.

Tissue Preparation and Tumor Detection

At necropsy, all fish were anesthetized in MS-222, weighed and measured. Scales, fins, and the caudal peduncle were removed, the body cavity opened ventrally, and the swim bladder punctured prior to fixation in Bouin's solution. After 24 hr fixation, the fish were dehydrated, infiltrated, and embedded in paraffin on their side so that sagittal sections could be cut from the left side. Three 5-6 μ m sections were kept from each fish (one at mid-eye

level, one just past the eye, and one in the midline), mounted on a single glass slide, and stained with hematoxylin and eosin. Organ accountability was good using this procedure. Tumor detection was based solely on observations on the three saved sections.

Statistics

Tumor incidence data were analyzed by logistic regression with categorical and/or continuous predictors in the Genmod procedure of SAS (SAS, 1996). For experiments with replicate tanks, there was no evidence of overdispersion ($p > 0.5$, all lack of fit tests) and residuals appeared consistent with the binomial error model. For the fry water bath exposure to DEN, the usual large-sample Chi-square p-value was quite close to 0.05, and for that case, an exact permutation p-value is also given as generated by StatXact version 3.1 (Mehta and Patel, 1995).

RESULTS

Note: Tables in this text are numbered as they appear in the attached carcinogenesis manuscripts (see Appendices). For more details, see attached manuscript preprints.

Summary of Neoplastic Responses

Dietary Exposure

DEN/DMN -- There were no differences in growth or behavior of the various experimental and control groups for both DEN and DMN during the 6-mo trial period. All ate equally well and responded normally to external stimuli. Mortalities were low except for unexplained losses in three tanks. No pathologic changes in the liver or other organs were detected by histologic examination after 26 wk (Tables 2.1 and 2.2).

Table 2.1. Carcinogenic response of zebrafish to 12-wk dietary DEN exposure

Lot	DEN dose ppm	Mortality %	Neoplastic response ^a	
			Inc.	%
1	Control	3	0/97	0
2	Control	2	0/98	0
1	500	7	0/93	0
2	500	15 ^b	0/85	0
1	1000	20 ^b	0/80	0
2	1000	2	0/98	0
1	2000	6	0/94	0
2	2000	0	0/100	0

^aFish necropsied 6 mo after start of carcinogen exposure

^bUnexplained loss of fish

Table 2.2. Carcinogenic response of zebrafish to 12-wk dietary DMN exposure

Lot	DMN dose ppm	Mortality %	Neoplastic response ^a	
			Inc.	%
1	Control	20 ^b	0/80	0
2	Control	1	0/99	0
1	500	5	0/95	0
2	500	0	0/100	0
1	1000	0	0/100	0
2	1000	0	0/100	0
1	2000	0	0/100	0
2	2000	0	0/100	0

^aFish necropsied 6 mo after start of carcinogen exposure^bUnexplained loss of fish

AFB₁ – Mortality was low in the control groups but increased with AFB₁ dose (Table 3.1). Among those that survived, there was little evidence of a DHEA effect on tumor incidence ($p=0.1520$, 1 df), but there was a significant effect due to AFB₁ dose ($p=0.0004$, 2 df). Over the three AFB₁ doses (10 to 30 ppm) there was a significant linear dose response ($p<0.0001$, logistic regression, 1 df) with no evidence of lack of fit to linear ($p>0.5$, 1 df). DHEA was not carcinogenic at the 444 ppm level when fed to control zebrafish.

Table 3.1. Effect of DHEA on mortality and tumor incidence when fed after dietary AFB₁ exposure^a

Dietary additions ^b		Lot	Mortality %	Neoplastic Response Inc. ^{c,d}	%
AFB ₁ ppm	DHEA ppm				
0	0	1	1	0/89	0
0	444	2	1	0/89	0
10	0	3	12	1/78	1
10	444	4	23	2/67	3
20	0	5	28	3/62	5
20	444	6	35	3/55	6
30	0	7	30	6/60	10
30	444	8	32	11/58	19

^aAbbreviations used: AFB₁ - aflatoxin B₁; DHEA - dehydroepiandrosterone

^bAFB₁ and DHEA were added to the modified purified casein diet, AFB₁ was fed for mo 1-3, DHEA for mo 4-9

^cNo. of fish with tumors/total No. of surviving fish

^dThere is little evidence of a DHEA effect on tumor incidence ($p=0.1520$, 1 df), but there is a significant effect due to AFB₁ dose ($p=0.0004$, 2 df)

MAM-Ac -- Table 4.1 summarizes the results of the dietary exposure of zebrafish to MAM-Ac. The fish tolerated all the doses well. Feeding behavior and growth were similar in all groups. Mortalities were slightly elevated at the highest dose. Among those that survived, there were highly significant differences in tumor incidence between treatments ($p<0.001$, 3 df). Among the three MAM-Ac doses (500 to 2000 ppm) the response increases with dose and the large increase between 1000 and 2000 ppm results in significant curvilinearity in the dose response ($p=0.0047$, quadratic term).

Table 4.1. Carcinogenic response of zebrafish to dietary MAM-Ac^a.

Lot	MAM-Ac dose ppm	Mortality %	Neoplastic response	
			Inc. ^c	% ^d
1	0	8	0/82	0
2	0	9	0/81	0
3	500	17 ^b	0/73	0
4	500	25 ^b	0/65	0
5	1000	7	5/83	6
6	1000	8	8/82	9
7	2000	11	23/79	29
8	2000	17	16/73	22

^aFish were fed MAM-Ac diets for 12 wk and terminated 24 wk after the start of the exposure

^bUnexplained loss of fish

^cNo. of tumor bearing fish/total No. of fish

^dThere were highly significant differences in tumor incidence between treatments ($p < 0.0001$, 3df)

The data comparing the response of zebrafish and medaka to dietary MAM-Ac at 1000 and 2000 ppm is presented in Table 4.3. As shown, the responses of the two species were very similar. There was no evidence of a difference between species of fish ($p > 0.5$, 1 df) or interaction lack of fit ($p > 0.5$, 1 df). The incidence of liver neoplasms was slightly higher in medaka than zebrafish, but medaka did not develop neoplasms in other tissues as did the zebrafish.

Table 4.3. Carcinogenic response of zebrafish and medaka to dietary MAM-Ac^{ab}

Species	MAM-Ac dose ppm	Mortality %	Neoplastic response	
			Inc. ^c	% ^f
Zebrafish	0	9	0/163	0
Zebrafish	1000	8	13/165	8
Zebrafish	2000	16	39/152	26
Medaka	0	0	0/100	0
Medaka	1000	5	6/95 ^d	6
Medaka	2000	28	20/72 ^e	28

^aFish were fed MAM-Ac diet for 12 wk then control diet for an additional 12 wk

^bData for zebrafish exposed to 1000 and 2000 ppm MAM-Ac were pooled from Table 4.1

^cNo. of tumor bearing fish/total No. of fish

^dTumors were diagnosed as follows: three cholangiocellular carcinomas, two hepatocellular carcinomas, three hepatocellular adenomas

^eTumors were diagnosed as follows: one hepatocellular adenoma, one cholangiocellular adenoma, seven hepatocellular carcinomas, nine cholangiocellular carcinomas, and two mixed carcinomas

^fThere was a highly significant difference between the two MAM-Ac doses (0.0001, df) but no evidence of a difference between the two species ($p > 0.5$, 1df)

MNNG -- No neoplasia was observed in zebrafish exposed to dietary MNNG.

DMBA -- No neoplasms were observed in the negative controls, DMSO carrier controls, or the 100 ppm DMBA exposed fish. Of the fish fed 500 ppm DMBA, 10% developed neoplasms; 11% of the fish fed 1000 ppm DMBA developed neoplasms.

Note: Data for the MNNG and DMBA groups were recently acquired. Statistical analysis and incorporation into manuscripts is in progress.

Fry Bath Exposure

DEN/DMN -- Cumulative mortality in the DEN experiment was higher than desirable and partially the result of an *Oodinium sp.* parasitic outbreak. However, there was also mortality associated with DEN exposure, especially at the highest dose (Table 2.3). For fish that survived, there was evidence of differences in neoplastic response between the four lots receiving DEN ($p=0.0509$, standard Chi-square approximation, 3 df) and ($p=0.0485$, exact permutation, 3 df). Over the four DEN doses (500 to 2000 ppm) there is a significant linear dose response ($p < 0.0134$, logistic regression, 1 df) with no evidence of lack of fit to linear ($p > 0.5$, 2df).

Table 2.3. Carcinogenic response of 14 day post-hatch zebrafish fry exposed to static water solutions of DEN for 24 hr, and terminated 12 mo later

Lot	DEN dose ppm	Mortality %	Neoplastic response	
			Inc. ^a	% ^b
1	Control	32	0/82	0
2	500	60	5/48	10
3	1000	52	7/58	12
4	1500	50	14/61	23
5	2000	98	2/3	67

^aNo. of fish with at least one neoplasm/total No. of fish

^bOver the four DEN doses, there is a significant linear dose response ($p < 0.0134$, 1df, logistic regression)

Mortalities occurred in the DMN experiment as well but were not dose responsive (Table 2.4). Among those that survived, there were highly significant differences in neoplastic response between lots ($p < 0.0001$, 5 df). There was increasing response with DMN dose until the highest dose which results in strong evidence of curvilinearity in the DMN dose response ($p < 0.0001$, 1 df quadratic term) and no evidence of lack of fit to quadratic ($p > 0.50$, 1 df).

Table 2.4. Carcinogenic response of 14-day post-hatch zebrafish fry exposed to static water solutions of DMN, and terminated 12 mo later

Lot	DMN dose ppm	Mortality %	Neoplastic response	
			Inc. ^a	% ^b
1	Control	1	0/119	0
2	250	17	0/100	0
3	500	30	1/84	1
4	1000	17	12/100	12
5	1500	22	25/94	27
6	2000	50	26/60	43
7	2500	33	31/81	38

^aNo. of fish with at least one neoplasm/total No. of fish

^bAmong survivors, there are highly significant differences in neoplastic response between tanks ($p < 0.0001$, 5df).

MAM-AC -- The results of this part of the study are included in Table 4.4. Both the mortalities and neoplastic response were dose related, but the overall response was low, only 12 and 14% at the two highest doses, respectively. Since neoplasms were observed only at the three highest doses, and all these doses produced treatment related mortalities, there appears to be a very narrow range between lethality and carcinogenicity with this exposure route.

Table 4.4. Carcinogenic response of 21-day post-hatch zebrafish fry exposed to static water solutions of MAM-Ac^a

Lot	MAM-Ac dose ppm	Mortality %	Neoplastic response	
			Inc. ^b	%
1	0	0	0/100	0
2	6.25	0	0/100	0
3	12.5	0	0/100	0
4	25	0	0/100	0
5	50	15	4/85	5
6	75	26	9/74	12
7	100	35	9/65	14

^aZebrafish fry were exposed to MAM-Ac solutions for 2 hr and terminated 12 mo later

^bNo. of tumor bearing fish/total No. of fish

MNNG -- In the zebrafish fry exposed to 0.5 ppm MNNG, 22% developed neoplasms.

DMBA -- In the sham control fish, 2 fish developed neoplasms (2%). In the DMSO controls, 1 neoplasm in a single fish (1%) was observed. In the 1.25, 2.5 and 5.0 ppm dose groups, 51%, 45% and 66%, respectively, of the fish developed neoplasms.

Note: Data for the MNNG and DMBA groups were recently acquired. Statistical analysis and incorporation into manuscripts is in progress.

Embryo Bath Exposure

DEN -- Zebrafish embryos were exposed only to DEN. The neoplastic response and mortality data are presented in Table 2.7. Treatment related mortalities and tumor incidences were lower, at comparable or higher doses, than for fry exposure to DEN. Mortalities differed significantly between treatments ($p < 0.0001$, 3 df). Over the three DEN doses

(1000 to 3000 ppm) there was a significant linear dose response ($p < 0.001$, 1 df logistic regression) with no evidence of lack of fit to linear ($p > 0.5$, 1 df). Among those that survived, there were highly significant differences in neoplastic response between treatments ($p = 0.0031$, 3 df). The treatment differences can be explained by a highly significant difference between the controls and all of the lots getting at least some DEN dose ($p = 0.0005$, 1 df). Among lots receiving DEN, there was no evidence of differences due to dose ($p = 0.3937$, 2df).

Table 2.7. Carcinogenic response of zebrafish, exposed to static DEN solutions as 60 hr embryos, for 24 hr, and terminated 12 mo later

Lot	DEN dose ppm	Mortality % ^c	Neoplastic response	
			Inc. ^a	% ^b
1	Control	51	1/73	1
2	Control	55	1/68	1
1	1000	50	5/75	7
2	1000	53	5/70	7
1	2000	67	6/50	12
2	2000	65	6/52	11
1	3000	75	3/37	8
2	3000	80	4/30	13

^aNo. of fish with at least one neoplasm/total No. of fish

^bAmong the lots receiving DEN, there was no evidence of differences due to dose ($p = 0.3937$, 2df)

^cMortalities differed significantly between treatments ($p < 0.0001$, 3df)

MAM-Ac -- Zebrafish exposed to MAM-Ac as embryos suffered high mortalities particularly in the early stages of the experiment (Table 4.6). This occurred in the controls, but there was also an obvious treatment effect as well. When an experiment is started with embryos, and losses are based on the initial number of embryos, mortalities run higher than if the initial number is based on fry or adults that have survived the more vulnerable stages of hatching, swimup, and the onset of feeding. At termination, there was no evidence of tissue damage that could have explained the earlier mortalities. Although there were few survivors at the highest dose, tumor incidences were the highest (11/20, and 12/17) of all the exposure routes tested. Among those that survived, there were highly significant differences in tumor incidence between treatments ($p < 0.0001$, 2 df). Over the three MAM-Ac doses (10 to 50 ppm) there was a significant linear dose response ($p < 0.0001$, logistic regression, 1 df) with no evidence of lack of fit to linear ($p > 0.5$, 1 df).

Table 4.6. Carcinogenic response of zebrafish, exposed to MAM-Ac for 12 hr as 72-hr embryos, and terminated 12 mo later

Lot	MAM-Ac dose ppm	Mortality %	Neoplastic response	
			Inc. ^a	% ^b
1	0	35	0/97	0
2	0	52	0/72	0
1	10	51	4/73	5
2	10	55	2/67	3
1	25	62	8/57	14
2	25	77	4/35	11
1	50	87	11/20	55
2	50	89	12/17	71

^aNo. of fish with tumors/total No. of fish

^bThere were significant differences in tumor incidence between treatments ($p < 0.0001$, 2 df)

MNNG -- Of the various exposure routes to MNNG, embryonic bath exposure resulted in the highest incidence of neoplasms. A 1% tumor incidence was observed in the negative controls, while the 1, 5 and 10 ppm doses produced 4.6%, 16.6% and 33% neoplasms, respectively.

DMBA -- Control fish had a tumor incidence of 1%, while the 0.25, 0.5 and 1.0 ppm groups had 2.5%, 12% and 5% tumor response, respectively.

Note: Data for the MNNG and DMBA groups were recently acquired. Statistical analysis and incorporation into manuscripts is in progress.

Embryo Microinjection

The fish from the MNNG experiment were only recently terminated. Preliminary observations indicate that about 10% of the fish have hepatic neoplasms, with hepatocellular adenoma as the predominant type. These data will be finalized in the near future. As previously stated, the AFB₁-microinjected fish have not been terminated yet.

Summary of Histopathology

For the 4 carcinogens DEN, DMN, AFB₁ and MAM-Ac, the primary responding organ was the liver, regardless of the carcinogen or the route of exposure (dietary, fry bath or embryo bath). The spectrum of neoplasms in the liver was also monotonous over the various carcinogens and exposure routes. Foci of cellular alteration (clear cell, eosinophilic and basophilic foci), hepatocellular and cholangiocellular adenomas, hepatocellular and cholangiocellular carcinomas, and occasional mixed hepatocellular and cholangiocellular adenomas and carcinomas were seen in all treatment protocols (see figures on pages 20-23; these figure numbers correspond to figure numbers in attached manuscripts).

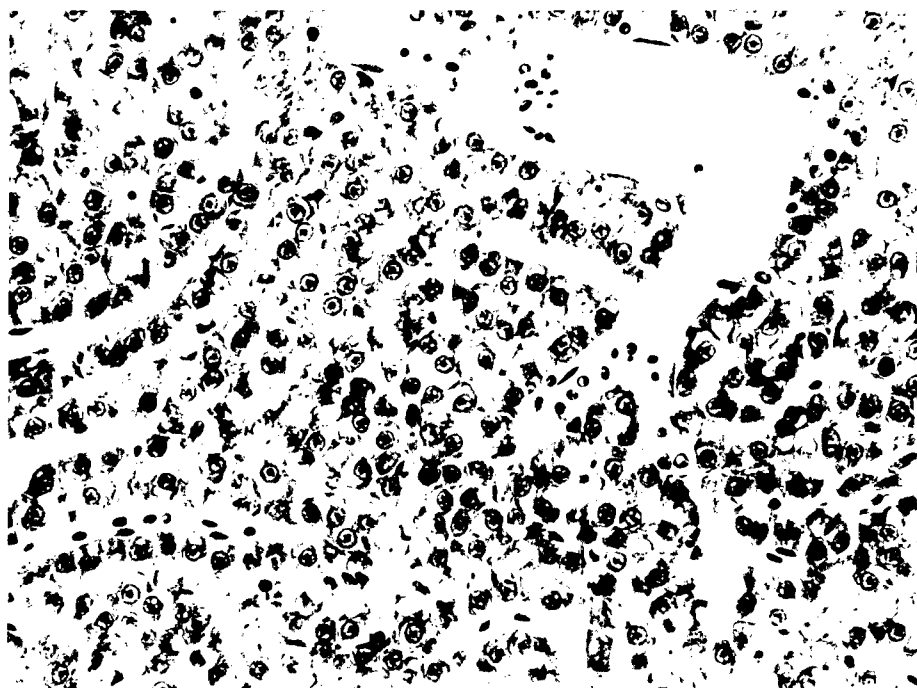


Fig. 4.1. Normal liver from control zebrafish. The two-cell-wide nature of the hepatic tubule cut longitudinally is clearly evident. Note also the uniform size and shape of hepatocyte nuclei, prominent single nucleolus, and moderate level of glycogen vacuolation. H&E, X544.



Fig. 4.2. An eosinophilic focus (upper right) and two clear cell foci in the liver of a zebrafish exposed to 75 ppm MAM-Ac in a fry water bath. An area of normal liver occurs between the foci. H&E, X340.

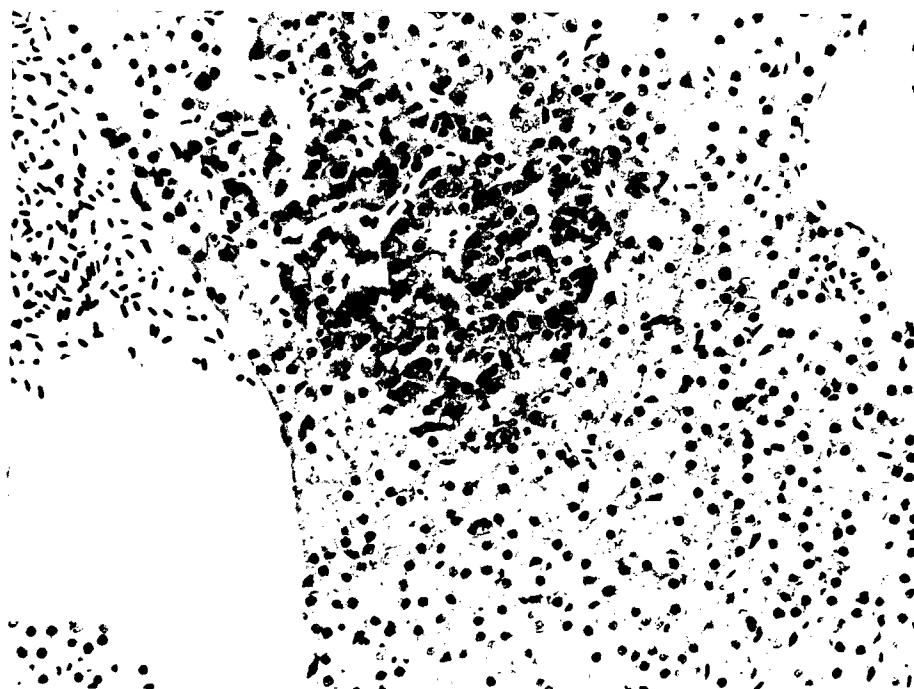


Fig. 4.3. A basophilic focus in the liver of a zebrafish exposed to 2000 ppm dietary MAM-Ac. Hepatic tubular structure is normal, but staining is distinctly basophilic. H&E, X340.

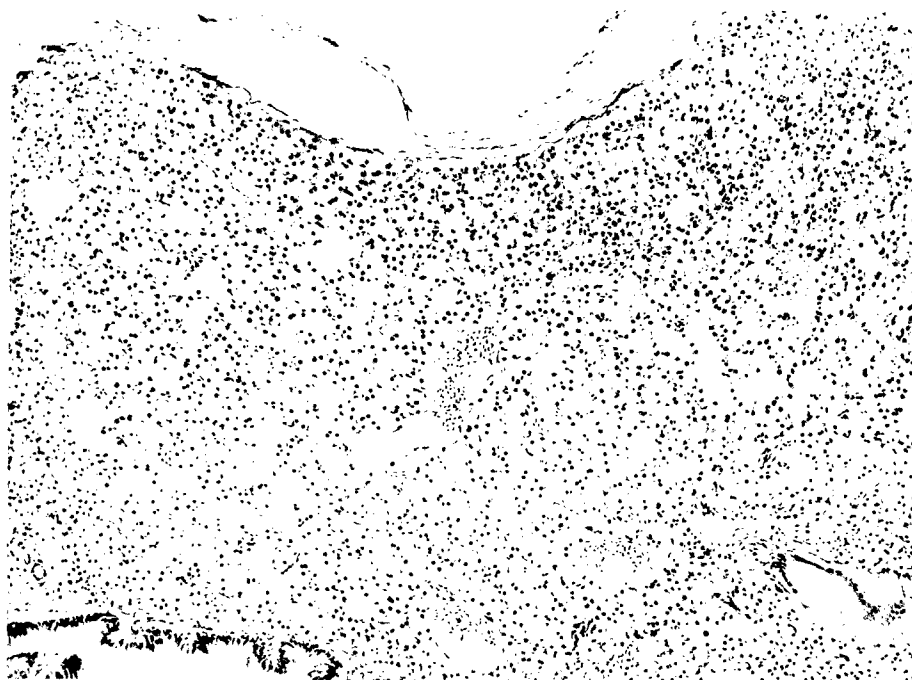


Fig. 4.4. An eosinophilic adenoma in a zebrafish exposed to 75 ppm MAM-Ac in a fry water bath. Normal liver is present at the far left. H&E, X136.

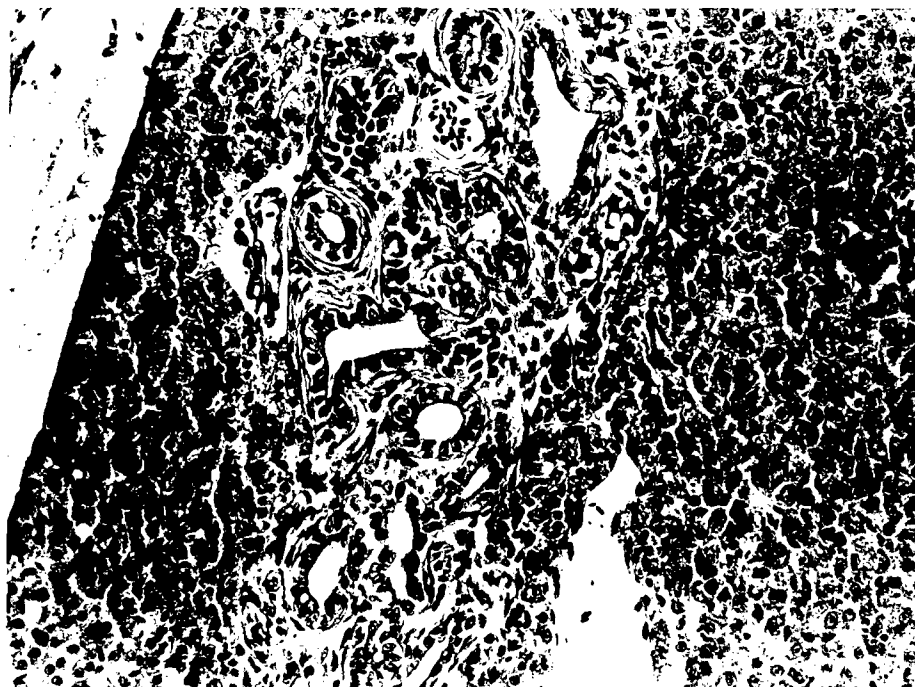


Fig. 3.6. A small cholangiocellular adenoma in the liver of a zebrafish fed 30 ppm AFB₁. The biliary ducts are normal in appearance and surrounded by connective tissue. H&E, X340.

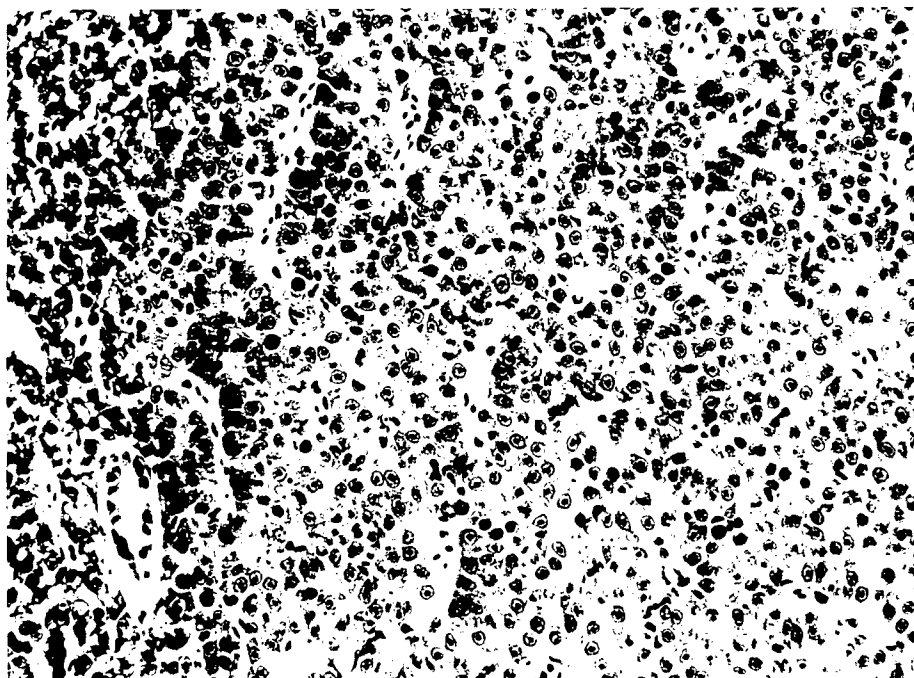


Fig. 4.6. A large hepatocellular carcinoma in a zebrafish exposed to 2000 ppm dietary MAM-Ac. Hepatic tubules are multiple cells wide between adjacent sinusoids, cells are deeply basophilic, devoid of glycogen and mitotic figures are common. Normal liver is on the left H&E, X340.

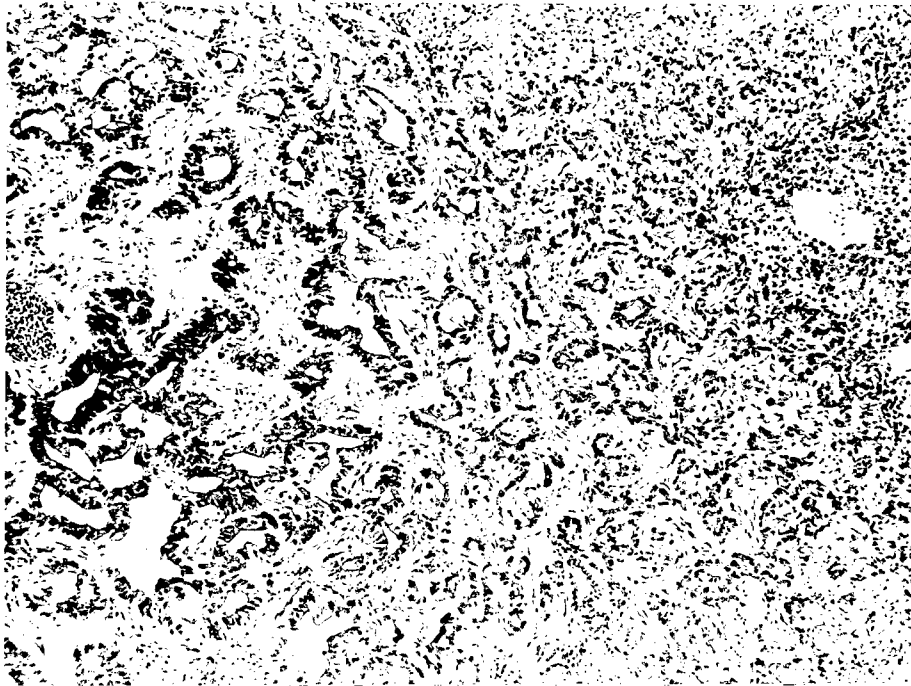


Fig. 4.11. A large cholangiocellular carcinoma from a zebrafish exposed to the 75 ppm MAM-Ac fry water bath. Note the branching biliary ducts and invasion of liver at upper right. H&E, X136.

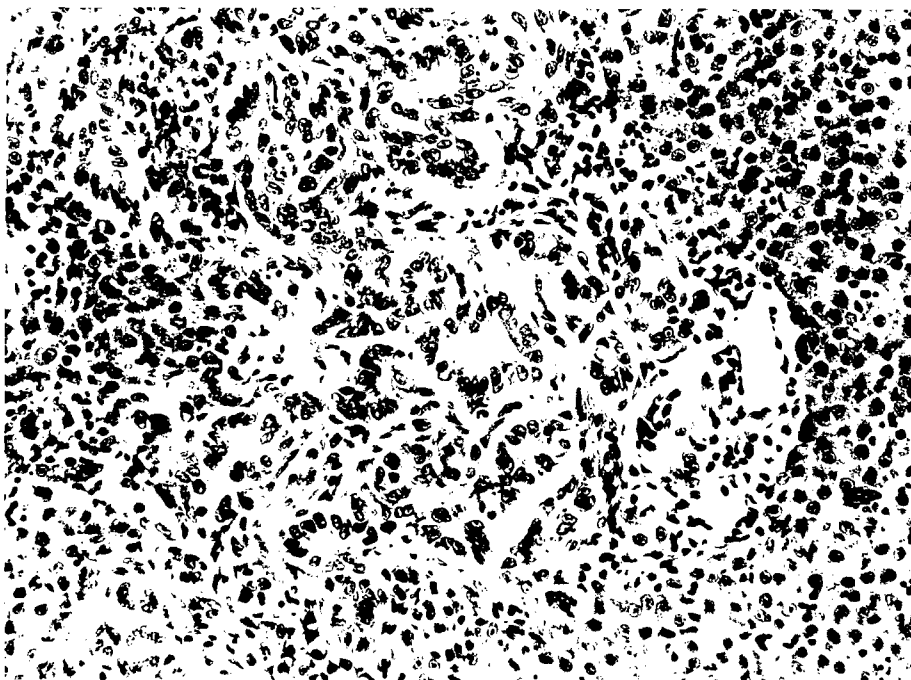


Fig. 4.13. A mixed carcinoma having hepatocellular (far right) and biliary components intermixed. The individual parts are similar those in either hepatocellular or cholangiocellular carcinomas. Zebrafish was exposed to 2000 ppm dietary MAM-Ac. H&E, X340.

Medaka exposed to dietary MAM-Ac produced only liver tumors, but also had frequent spongiosis hepatitis lesions, a lesion not seen in zebrafish in our studies. The hepatic tumors in medaka were similar in type to those observed in zebrafish.

Neoplasms in other organs varied from carcinogen to carcinogen and with exposure route. In general, these other neoplasms were rare single occurrences, but the total number of such neoplasms was usually greatest in the embryo exposed animals, intermediate in fry exposed animals, and least in dietary exposed animals. For comparative purposes, these lesions will be listed by exposure route and carcinogen, but will not be illustrated.

Dietary Exposure

DEN/DMN -- No neoplasms of any kind were observed with this route of exposure.

AFB₁ -- There was a single ductal adenocarcinoma in the pancreas of one zebrafish.

MAM-Ac -- There was a single adenocarcinoma in the intestine of one zebrafish, a leiomyosarcoma in the intestine of two separate zebrafish, a single fibrosarcoma in the body wall of one zebrafish, and a single adenocarcinoma in the pancreas of one zebrafish. All these lesions occurred in the highest (2000 ppm) dose group.

MNNG -- No neoplasms were observed with this route of exposure.

DMBA -- Mucosal adenoma or adenocarcinoma of the intestine occurred in 2% and leiomyoma or leiomyosarcoma occurred in 1% of all zebrafish that received dietary DMBA.

Fry Bath Exposure

DEN -- No non-hepatic neoplasms were observed.

DMN -- There was a single hemangiosarcoma in the liver of one zebrafish, and a single leiomyosarcoma in the intestine of one zebrafish.

AFB₁ -- Fry bath exposure to AFB₁, not otherwise reported here, did not produce tumors at any site.

MAM-Ac -- There was a single osteosarcoma in the gill of one zebrafish in the 75 ppm group.

MNNG -- Blood vessels and testes were the primary target organs in zebrafish treated as fry with MNNG. Hemangioma or hemangiosarcoma occurred in 9% of all zebrafish treated as fry with MNNG. Seminomas were present in 7% of all zebrafish treated as fry with MNNG. Small numbers of other types of epithelial and mesenchymal neoplasia occurred in a variety of organs in zebrafish treated as fry with MNNG.

DMBA -- In the sham control group, two fish developed adenocarcinomas of the exocrine pancreas. One fish in the DMSO control developed an adenocarcinoma of the intestine. Liver was the primary target organ in zebrafish treated as fry with DMBA, with gills and blood vessels the second and third most frequently affected organs. Hepatic neoplasia occurred in 31%, chondroma or chondrosarcoma in the gills occurred in 20%, and hemangioma or hemangiosarcoma was present in 4%. Furthermore, zebrafish treated as fry with DMBA exhibited the widest variety of target organs and broadest spectrum of histologic types of neoplasia of all of our carcinogen experiments with zebrafish. In addition to liver, epithelial neoplasia occurred in intestine, pancreas, thyroid and testis. In addition to cartilage and vascular neoplasms, mesenchymal neoplasia occurred in smooth and skeletal muscle, nerve sheath, connective tissue, and lymphoid tissue. In all, 28% of zebrafish treated as fry with DMBA showed mesenchymal neoplasia. Finally, 2% of zebrafish treated as fry with DMBA exhibited primary brain neoplasms, present as neuroblastomas.

Embryo Bath Exposure

DEN -- Neoplasms of the following types were observed: an adenocarcinoma of the intestine, two chordomas of the spine, a fibrosarcoma of the operculum, a chondrosarcoma of the gill, an ultimobronchial body adenoma, a leiomyosarcoma of the intestine, and an inverted papilloma of the nares.

DMN -- No fish were exposed to DMN as embryos.

AFB₁ -- No tumors of any type were observed from embryo bath exposure to AFB₁.

MAM-Ac -- Two fish developed testicular seminomas in the 10 ppm groups. In the 25 ppm group, we observed a testicular seminoma, an intestinal leiomyosarcoma, a chondrosarcoma of the gill, and an exocrine pancreatic adenoma. In the 50 ppm groups, we observed the following neoplasms: a ductal adenoma in the pancreas, an osteochondroma in the sclera of the eye; a neurofibroma in the muscle of the trunk region, a medulloepithelioma in the retina of the eye, a renal adenoma, a neurofibrosarcoma of the spine, a neuroblastoma of the brain, a rhabdomyoma of the heart, and single hemangiomas in the pharynx, ovary, tail region and gill.

MNNG -- The principal target organs were liver and testis. Seven percent of all carcinogen-exposed fish had hepatic neoplasia, with hepatocellular adenoma the most prevalent hepatic neoplasms. Seminomas occurred in 6% of all zebrafish exposed to MNNG as embryos. Mesenchymal neoplasia (including chondroma, hemangioma, hemangiosarcoma, leiomyosarcoma and rhabdomyosarcoma) occurred in 2% of all zebrafish treated as embryos with MNNG.

DMBA -- Liver was the primary target organ in zebrafish treated as embryos with DMBA. Hepatic neoplasia occurred in 4% of zebrafish treated as embryos, with hepatocellular adenoma as the primary tumor type. In addition, a single neurofibroma located in the kidney, a single neurofibrosarcoma of the peritoneum, and a single seminoma were observed.

DISCUSSION

Dietary exposure of aquarium fish to carcinogens has been used sparingly in comparison to water exposures (Khudoley, 1972; Sato *et al.*, 1973; Pliss and Khudoley, 1975; Hatanaka *et al.*, 1982). Most of these experiments have used water insoluble compounds such as aflatoxins, heterocyclic amines, azo dyes, or polycyclic aromatic hydrocarbons, and have produced significant numbers of hepatic neoplasms in both guppies and medakas. Sato *et al.* (1973), however, did feed DMN to guppies at 4,800 ppm for 13 mo, and saw only a minimal response, 2 of 20 fish had what were described as hyperplastic nodules in the liver. The total lack of response to DMN and DEN after 12 wk of exposure to high doses of these compounds may be the result of several factors: 1) insufficient exposure time and/or dose. The only dietary exposures of fish to these carcinogens, for comparison, have been the study by Sato *et al.* (1973), where both a higher dose and longer exposure to DMN gave a minimal response, and with rainbow trout, in which lower doses but longer exposure times of either 9 or 12 mo gave high incidences of liver tumors (Grieco *et al.* 1978; Hendricks *et al.*, 1994). The shorter exposure time used in the current experiment was based on the higher doses used compared to the trout studies, the shorter life span of zebrafish compared to trout which could translate into a shorter response time, and facility demands which prevented longer term exposures, 2) age of the fish when exposure began. The guppies used by Sato *et al.* (1973) were less than one mo old. In rainbow trout, we have observed decreasing sensitivity to carcinogen exposure with increasing age (unpublished observations), thus the fact that our zebrafish were two mo old and near sexual maturity, may have reduced their sensitivity, and 3) possible pharmacokinetic differences in zebrafish that limit absorption and distribution of dietary-delivered carcinogens, specifically nitrosamines. The lack of a stomach in zebrafish may alter digestion, absorption, and delivery of carcinogens to the liver as compared with the rainbow trout, a fish that responds with high sensitivity to dietary carcinogen exposures.

The spectrum of hepatic neoplasms produced by DEN and DMN in the water-borne exposures of both fry and embryos, were similar to those reported by authors exposing several other species of small fish to DEN (Stanton, 1965; Schultz and Schultz, 1982a; Parland and Baumann, 1985; Couch and Courtney, 1987; and Bunton, 1989). One lesion that has been reported in medaka and sheepshead minnows, in response to water-borne DEN, is spongiosis hepatitis (Hinton *et al.*, 1984; Couch and Courtney, 1987). This lesion has not been seen thus far in any of our zebrafish samples.

Perhaps the most interesting result from the exposures of zebrafish to nitrosamines is the unusual or rare tumor types observed in the fish exposed to DEN as embryos. Although few in number, the chordomas of the spine, the chondrosarcoma of the gill and the ultimobranchial adenoma are tumor types that have not been previously described from DEN exposures to aquarium fish. The only other known embryo exposure of aquarium fish to a carcinogen was reported by Klaunig *et al.* (1984). They exposed medaka to DEN at concentrations of 25, 50, and 100 ppm for 10 days, and found liver tumors in 4, 15, and 43% of the fish, respectively, after six months. It is not possible to compare the relative sensitivities of medaka and zebrafish in these two studies. However, it appears that medaka

were more responsive to hepatocarcinogenesis, but no other tumor types were reported. They used lower doses for a longer time, something we could not do with zebrafish, since the entire developmental period from fertilization to hatch is only 96 hr.

The overall low response of zebrafish to the hepatocarcinogenicity of strong carcinogens such as DEN and DMN, may be due to inherent genetic factors in zebrafish and other members of the Cyprinidae family. Other members of that family have demonstrated dramatic resistance to carcinogenesis in both controlled laboratory experiments (*Pimephales promelas*, the fathead minnow) and highly polluted natural environments (*Cyprinus carpio*, the common carp) (Hawkins *et al.*, 1988a). It is possible that fish of this family have specific digestive, pharmacokinetic, metabolic, DNA repair, tumor suppressor gene, or other mechanisms that provide protection against carcinogenesis.

The results of the exposures to AFB₁ confirm previous reports in the literature and preliminary experimental data generated in our laboratory, that zebrafish are surprisingly resistant to the carcinogenicity of AFB₁. The work of Troxel *et al.* (see appended manuscript; see Metabolism section) has investigated the metabolism and DNA-binding of AFB₁ in zebrafish and provides some hypotheses for this resistance. Troxel *et al.* (see appended manuscript) found that zebrafish begin to rapidly excrete the metabolite, aflatoxicol (AFL), within 5 min of an intraperitoneal injection of AFB₁, and within 24 hr, 47% of the administered radioactivity had been excreted into the water. After 18 hr, AFL-glucuronide became the major metabolite excreted. Thus, zebrafish have an extremely fast-acting cytosolic reductase system that begins generating AFL within 5 min and have the ability to excrete this metabolite directly into the water, presumably through the gills. Later, phase II metabolism takes over and AFL-glucuronide is excreted, probably through the urine. Both of these mechanisms could function to reduce the available AFB₁ that could be metabolized to the epoxide for binding to DNA, and provide protection against AFB₁ carcinogenesis. Supportive of this hypothesis is the 4-fold lower DNA binding of AFB₁ in zebrafish in comparison with rainbow trout (Troxel *et al.*, see appended manuscript). They also found that female zebrafish bind approximately twice as much AFB₁ to liver DNA as males. We were interested to see if this increased DNA binding in females would result in a higher incidence of neoplasms in females than males. However, when we noted the sex of the tumor-bearing fish, there was no evidence of a sex effect. Of the 26 total tumor-bearing fish, 14 were males and 12 were females. At the high dose of AFB₁, three males and three females had tumors, and when DHEA was added, six males and five females bore tumors. Buchmann *et al.* (1993) showed that the cytochrome P450 isozyme (CYP2K1), thought to be responsible for bioactivation of AFB₁, is constitutively expressed in zebrafish, providing a basis for the response that we do observe.

Another variable that effects the sensitivity of rainbow trout to carcinogens is age. We have found that young rainbow trout (embryos, and small fry) are much more susceptible to AFB₁ carcinogenesis than are older fish (Hendricks, unpublished observations). The zebrafish used in this feeding trial were adults or subadults (2-mo-old) when the dietary exposure began, and thus they could possibly have been more responsive if they had been younger. However, the results of Troxel *et al.* (see appended manuscript) were also obtained on adult fish, so their results do directly apply to ours.

Although there appeared to be some slight effect of DHEA as a promoter, its effects were much less than in rainbow trout, and DHEA demonstrated no initiating activity at a dose that was clearly carcinogenic to rainbow trout (Orner *et al.*, 1995). In rodents, DHEA is a peroxisomal proliferator, and it is thought to exert its carcinogenic properties through poorly understood mechanisms involving peroxisome proliferation (Rao *et al.*, 1992). In rainbow trout, however, which respond poorly to peroxisome proliferators, carcinogenesis and promotion occurred in the absence of this mechanism (Orner *et al.*, 1995). Orner *et al.* (1995) suggested that the promotional mechanism of DHEA may be due to its conversion into excess estrogens, which are promoters of carcinogenesis in rainbow trout (Nunez *et al.*, 1989). We do not know how zebrafish respond to peroxisome proliferators, but would assume that their response would be more like trout than rodents.

Our previous experiments with zebrafish have shown a trend towards relative insensitivity to the carcinogenicity of nitrosamines and AFB₁; however, we had not conducted any side-by-side comparisons with other species. In this study, dietary exposure of zebrafish and medaka to the same MAM-Ac-containing diets revealed an almost identical carcinogenic response in the two species. Khudoley (1984) compared zebrafish and guppies with respect to nitrosamine carcinogenicity and concluded that zebrafish were more sensitive than guppies. Hawkins *et al.* (1985) compared seven species of aquarium fish and concluded that the medaka and guppy were both desirable models but did not make any quantitative comparisons. Additional species to species comparisons in the same laboratory would be useful for ranking the various species with respect to sensitivity.

Route of exposure can have a pronounced effect on target organ response, especially with a direct-acting carcinogen (Hendricks *et al.*, 1980; Hendricks *et al.*, 1995; Orner *et al.*, 1995). For DEN, DMN, AFB₁ and MAM-Ac, the effect was not as variable as expected. With all three routes of exposure, the liver was the major target organ, and the response at other organ sites was so low and scattered, there was no secondary target organ. With the embryo water exposure to MAM-Ac, there was also a relatively narrow dose range in which carcinogenicity occurred without mortality being excessively high.

There was a distinct difference between zebrafish and medaka with respect to the occurrence of spongiosis hepatis. This putative neoplasm of perisinusoidal cells or cells of Ito occurs frequently in carcinogenesis studies with medaka (Hinton *et al.*, 1984) and sheepshead minnow (Couch and Courtney, 1987), but we have never observed it in our carcinogenesis studies with zebrafish. It also has never been described in rainbow trout (Hendricks, unpublished observations).

Although other carcinogens need to be tested with the zebrafish, there are several characteristics of this species that would limit its usefulness as an environmental monitor or even as a laboratory model. First, there is the apparent lack of responsiveness to certain carcinogens. For this reason this species may not detect carcinogenic problems in the environment as well as a more sensitive species might. Second, and probably most important, is its inability to survive at temperate water temperatures, limiting its use to tropical or sub-tropical waters only. We have found that temperatures below 10°C completely immobilize zebrafish, and their activity is greatly reduced between 10 and 15°C.

For prolonged periods of exposure, water temperatures would probably need to be 18°C or higher for zebrafish to sustain normal bodily functions. Third, the location of the zebrafish liver, entwined around the intestine, makes the removal of liver tissue for metabolic studies very difficult and time-consuming. On the other hand, this species is easy to rear, produces eggs all year long, and has a very short development time (96 hr). Although zebrafish have proven to be advantageous for other biological research, we believe their negative characteristics outweigh their positive ones for carcinogenesis. Perhaps this is why no one has perpetuated Stanton's early work with zebrafish. These features may have been known but were never discussed.

METABOLISM STUDIES (Objective 4)

The aim of objective 4 was to characterized procarcinogen metabolism, DNA adduction, and DNA repair in zebrafish. Aflatoxin B₁ is a potent hepatocarcinogen which has been extensively characterized in the rainbow trout model. This mycotoxin is a secondary metabolite produced primarily by the molds *Aspergillus flavus* and *Aspergillus paraciticus*, and frequently contaminates improperly stored foodstuffs, such as corn and peanuts. Rainbow trout are currently the most sensitive species known to the carcinogenic effects of AFB₁. We chose this carcinogen first for the metabolism and DNA-adduction studies in zebrafish because there is an extensive knowledge base on the metabolic profile of AFB₁ in many species, and we could compare the sensitivity of the zebrafish to the most sensitive species known.

We then characterized cytochrome P4501A (CYP1A) induction in zebrafish following exposure to the commercial PCB mixture Aroclor 1254, the synthetic flavone β -naphthoflavone (β NF), and the most potent inducer known, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Many groups are investigating the use of CYP1A enzyme induction in fish as a biomarker of exposure to certain compounds commonly found in the environment, such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and halogenated dibenzo-*p*-dioxins and dibenzofurans. The enhanced metabolic capabilities following exposure to one of the many CYP1A-inducing agents may result in altered metabolism of other xenobiotics including procarcinogens such as AFB₁. After characterizing CYP1A induction in zebrafish, we subsequently investigated the influence of CYP1A induction by TCDD on AFB₁ metabolism and DNA-adduction.

Details of these studies are provided in the two appended manuscripts by Troxel *et al.*; the following report summarizes the salient features of this work.

METHODS

Adult (sexually mature) zebrafish were used for all experiments.

In Vivo Metabolism of [³H]AFB₁

To investigate AFB₁ metabolism, female zebrafish were i.p. injected with [³H]AFB₁. Following injection, fish were rinsed to remove skin contamination and placed in individual beakers of water. At various time points, the fish were moved to fresh beakers of water, the water samples were collected for metabolite analysis, and an aliquot was taken for tritium counting. The metabolites were extracted from the water samples using C₁₈ extraction disks, and the metabolic profile was determined by HPLC analysis.

Hepatic AFB₁-DNA Adduction

To investigate AFB₁-DNA adduction, male and female zebrafish were i.p. injected with [³H]AFB₁, rinsed, and placed in water. Livers were sampled 24 hours after injection

for the dose-response experiment, and up to 21 days after injection for the time course investigation. The DNA was extracted and purified from the liver samples, the DNA concentration was determined by microfluorometry, and the DNA was hydrolyzed and counted to determine total DNA binding.

In Vitro AFB₁ and AFM₁ Metabolism Assay

The assays were carried out with juvenile trout, or female or male zebrafish liver homogenates.

CYP1A Induction

CYP1A response in female zebrafish was investigated following exposure to Aroclor 1254, β NF, or TCDD. Aroclor 1254 or β NF were administered in the diet or via i.p. injection, and TCDD was only administered in the diet. CYP1A induction was assessed using the 7-ethoxyresorufin-O-deethylase (EROD) activity as a measure of catalytic activity, and antibody detection of protein (by western blotting) to quantify protein levels.

TCDD Modulation of AFB₁ Metabolism and Hepatic DNA-Adduction

To determine the influence of TCDD-pretreatment on AFB₁ metabolism and DNA-adduction, fish were fed control or TCDD-containing diet for 3 days, and then injected with carrier vehicle or [³H]AFB₁ on the fourth day. Metabolic profiles and AFB₁-DNA binding were determined as before. The metabolites aflatoxin M₁ (AFM₁) and aflatoxicol-M₁ (AFLM₁) were unable to be resolved from one another on HPLC, and are therefore grouped together.

RESULTS

Hepatic AFB₁-DNA Adduction

Fish i.p. injected with 50-400 μ g [³H]AFB₁/kg body weight displayed a linear dose-response for hepatic DNA binding at 24 hours. AFB₁-DNA adduct levels showed no statistical difference over the period from 1 to 21 days after injection, suggesting poor adduct repair in this species. Female fish had a 1.7 fold higher level of DNA binding than males ($p < 0.01$). An *in vitro* AFB₁ metabolism assay verified that zebrafish liver extracts support AFB₁ oxidation to the 8,9-epoxide proximate electrophile, the intermediate responsible for DNA-adduction.

A useful method for comparing DNA binding between species is the CBI (cumulative binding index, units of μ mol chemical bound/mol DNA/mmol chemical administered/kg body weight). A composite CBI calculated from the DNA-binding experiments in zebrafish is approximately 70,600 in female zebrafish and 35,000 for males. For comparison, the CBI for rat and Japanese medaka is approximately 10,000 and 13,000, respectively, and is 240,000 for trout (Toledo *et al.*, 1987, and references therein). From these data, zebrafish

exhibit approximately a 4-fold lower capacity for DNA adduct formation compared to trout, and a 5-fold higher capacity than rat and medaka.

In Vivo Metabolism of [³H]AFB₁

The results demonstrate that zebrafish have the capacity for both Phase I and Phase II metabolism of AFB₁. Early excretion kinetics of [³H]AFB₁ were evaluated by measuring the amount of radioactivity recovered in water within a 24 hour time period after i.p. injection. By 24 hours, 47% of the radioactivity administered to the zebrafish was recovered in the water. As is typical of other fish studied, such as the Japanese medaka and rainbow trout, the major *in vivo* metabolites excreted into water were aflatoxicol (AFL) and AFL-glucuronide, followed by unreacted AFB₁. Identification of these peaks was based on retention times of known aflatoxin standards generated in our lab. AFL appeared as early as 5 minutes after injection, whereas AFL-g was a significant metabolite after 18 hours.

CYP1A Induction

Aroclor 1254 fed at 500 ppm for 1 to 9 days or intraperitoneal (i.p.) injection of 75-200 mg Aroclor 1254/kg body weight failed to induce CYP1A protein or associated (EROD) activity. Dietary β NF at 500 ppm for 3 or 7 days induced CYP1A protein and EROD activity approximately 3-fold above controls. A single i.p. injection of 150 mg β NF showed maximal induction of CYP1A protein and EROD activity by 24 hours, both of which decreased rapidly during the next 6 days. A more refined study showed maximum CYP1A protein levels between 24 and 36 hours, while EROD activity had an observable peak at 16 hours. CYP1A and EROD activity showed dose-responsiveness following single i.p. administration of 25, 50, 100, or 150 mg β NF/kg body weight. Dietary exposure to 0.75 ppm TCDD for 3 days also significantly induced CYP1A.

TCDD Modulation of AFB₁ Metabolism and Hepatic DNA Adduction

The amount of radioactivity recovered in the water over 24 hours did not significantly differ between treatment groups. However, the actual metabolic profile was different between the two groups. The metabolic profile of the AFB₁ control group was similar to that observed in the earlier metabolism study. The major metabolites excreted into water were again AFL, AFL-glucuronide, and parent AFB₁, and the total recoveries of each metabolite were also consistent with the previous experiment. The AFB₁ metabolites excreted by the TCDD pre-treated group consisted of AFL, AFB₁, and AFL-g, but additionally included AFM₁/AFL-M₁ and the glucuronide of AFL-M₁. It is believed that CYP1A1 is the enzyme responsible for production of AFM₁, and these results are consistent with this hypothesis. EROD assays and western blotting of protein verified that CYP1A was not induced in the control group, but was induced in the TCDD-treated group.

The *in vivo* hepatic AFB₁-DNA adduction at 24 hours after i.p. injection of [³H]AFB₁ was determined to be almost 4-fold higher in the TCDD-treated group than in the control group. To further investigate the cause of this notable difference in adduction levels, *in vitro* AFB₁ and AFM₁ metabolism studies were conducted. The results from these metabolism

experiments showed no statistical difference between the control or TCDD-treated groups in their ability to bioactivate AFB₁ or AFM₁ to the respective reactive intermediate. There was also no statistical difference between the two groups in their capability of metabolizing AFB₁ to the primary metabolite AFL. However, the TCDD-treated group did have a 22-fold increase ($p=0.0001$) in their capability to metabolize AFB₁ to AFM₁. The increased binding could be related to the increased production of AFM₁ and/or AFL-M₁, and further activation to a DNA binding species. The *in vitro* AFM₁ metabolism assay did demonstrate that zebrafish are quite capable of bioactivating AFM₁ to a reactive intermediate capable of binding glutathione and forming an adduct.

DISCUSSION

These studies show that zebrafish are efficient at metabolizing AFB₁ to both phase I and phase II metabolites, including bioactivation of AFB₁ to a reactive intermediate capable of binding to DNA, as shown by the *in vitro* AFB₁ metabolism assay and confirmed in the *in vivo* hepatic DNA-binding experiments. These data suggest that zebrafish should be fairly sensitive to the carcinogenic effects of AFB₁. However, we have demonstrated that zebrafish are actually resistant to AFB₁-induced carcinogenesis when the carcinogen is administered in the diet or by static water bath (in this report). Hepatic DNA-adduction studies conducted following a dietary exposure to [³H]AFB₁ also showed only low levels of adducts (unpublished results). These results suggest a difference in absorbance and distribution of dietary AFB₁ compared to i.p. administration. Detailed pharmacokinetic studies will be needed to test this hypothesis. It seems that if one can get the carcinogen to the target organ, zebrafish are quite capable of bioactivating AFB₁.

Zebrafish CYP1A responded to the common CYP1A inducers β NF and TCDD, but surprisingly, there was no measurable response following exposure to Aroclor 1254, a commercial mixture of PCBs that is an effective inducer in trout and other species. A possible explanation for this enigma is that perhaps zebrafish possess a more selective agonist binding site than other species. Cloning and expression of the zebrafish Ah receptor would allow this hypothesis to be examined.

The modulation of AFB₁ metabolism with a more potent CYP1A inducer, in this case TCDD, was consistent with experiments conducted by other scientists. Due to increased levels of CYP1A, the metabolic profile shifted to formation of AFM₁ and AFL-M₁-g, the metabolite and its glucuronide conjugate typically associated with CYP1A. The 4-fold increase in hepatic DNA binding in the TCDD-treated group could not be explained by an enhanced capacity for bioactivation of AFB₁. It is postulated that AFM₁ is also bioactivated to a reactive intermediate that can bind to DNA. These results show that environmental exposure of fish species to modulators such as TCDD can indeed alter their response to genotoxic agents to which they may also be exposed.

GENETICS STUDIES (Objective 5)

The overall aims of technical objective 5 were to apply cellular and molecular genetics to investigate carcinogenesis at the gene level in normal and transgenic zebrafish. Specific objectives were to explore the role of oncogene activation in zebrafish carcinogenesis, to develop diploid cell lines and construct appropriate plasmid vectors for gene transfer studies, and to attempt to establish zebrafish carrying transgenes central to the cancer process. We emphasized that these represented long-term and challenging goals, only some of which were expected to be met within the support period of this project.

At the gene level, the two most common genetic lesions in human and experimental animal tumors involve mutations within the *ras* proto-oncogene family and the *p53* tumor suppressor gene (Bos *et al.*, 1988; Greenblatt *et al.*, 1994). We have also reported that oncogenic mutations in the *Ki-ras* proto-oncogene occur commonly in tumors from carcinogen-treated rainbow trout (Bailey *et al.*, 1996). An initial aim was to isolate and establish the DNA sequence of an expressed *ras* gene from zebrafish, and eventually to examine tumor DNA for evidence of transforming mutations in this gene. During the course of our work the importance of *p53* mutations in cancer became evident, and we also began an investigation of this most prominent tumor suppressor gene in zebrafish. We succeeded in cloning and sequencing zebrafish *ras* and *p53*, and to characterize their expression during embryo development. Two appended manuscripts (Cheng *et al.*) provide detailed methods and results, which will be only briefly outlined here.

The establishment of cell lines that display long term growth in culture yet retain diploid karyotype and full genetic competence has only recently become feasible. Such lines are highly useful for the study of xenobiotic metabolism, gene regulation, and for gene transfer. Under technical objective 5 we have established diploid lines from zebrafish embryos and several additional cell lines, have conducted initial gene transfer experiments, and have used these lines to examine regulation of CYP 1A regulation as a complement to Objective 4. Four appended publications (Miranda *et al.*, Collodi *et al.*) describe this work in detail.

A long-term goal was to develop zebrafish lines with germ-line transmission of transgenes pertinent to the process of cancer and its initiation by mutational processes. We envisioned two basic approaches: 1) construction of genetically competent cell lines selected for integration and expression of transgenes of interest, which could then be injected into fertilized embryos to produce mosaic founder lines; and 2) traditional production of founder lines by direct introduction of foreign DNA into fertilized embryos via microinjection or electroporation. Most recent attention has been given to transfer of a gene cassette for high sensitivity mutation detection in zebrafish, first with cells in culture. Development of the assay is based upon the successful stable transfection of zebrafish liver cells in culture with a lambda phage-based shuttle vector which carries marker gene sequences that are selectable in *E. coli*. Stable clonal lines carrying the shuttle vector can be exposed to various potential mutagens, the shuttle vector rescued from the cellular DNA by virtue of the lambda *cos* sites, and the frequency of mutational events scored in a bacterial system. The actual mutational event may then be identified by sequencing the marker gene region if desired.

This rationale is very similar to that employed in the transgenic mouse mutagenicity systems available from Stratagene and Hazelton. The lambda shuttle vector used in these experiments has been described (Brunelli and Pall, 1994). This construct has three main advantages over similar lambda shuttle vectors employed in the transgenic mouse mutagenicity assays. First, the test vector has been engineered to carry *loxP* sites, targets for site-specific recombination mediated by the Cre protein. Upon infection of an *E. coli* lysogen expressing *cre*, the plasmid is excised out of the lambda backbone to express whatever marker it carries in *E. coli*. Plasmid-based expression is much more reliable and efficient than lambda-based expression. Secondly, the construct contains selectable markers such that identification and quantitation of mutants will rely upon selection in addition to screening. Selection is much more efficient than just screening. Finally, the plasmid part of the construct contains sequences for detecting both forward and reverse mutations.

METHODS

Cloning, sequencing, expression of N-ras and p53

Zebrafish *ras* and *p53* gene sequences were sought by screening of two cDNA libraries. One of these was a CDNA library from the whole adult zebrafish provided by Dr. Philip McFadden at Oregon State University. We also constructed a CDNA library magnetically enriched for *ras*-related sequences from total zebrafish RNA using the Biomag Streptavidin system (Perseptive Diagnostics, Cambridge MA). To obtain *ras*, libraries were screened with an RT-PCR probe containing a 180 bp zebrafish *ras* fragment, which was obtained by PCR using primers based on Trout *Ki-ras* sequence. Positive plaques were revealed by chemiluminescent detection (Boehringer Mannheim, Indianapolis IN), and were identified only from the enriched library.

To screen for zebrafish *p53*, a non-radiolabeled probe based on conserved region IV of the rainbow trout *p53* gene, was amplified via polymerase chain reaction (PCR) by p53 E7 and cE7 primers, using the rainbow trout *p53* cDNA clone as template for the incorporation of digoxigenin-11-dUTP. This probe was used to screen the whole cDNA library, and a nearly full-length 1777 bp insert was isolated. To obtain a full-length clone, the cDNA library was re-screened using a random-primed, radiolabeled probe (Rediprime, Amersham) was generated from a PCR product from this 1777 bp insert.

The expression of *ras*, *p53*, *myc*, and the homologue of the murine *T* gene was carried out by Northern analysis of RNAs isolated at various stages of embryogenesis following egg fertilization, as detailed in the appended manuscripts.

Zebrafish cell lines

Proliferating zebrafish cultures were established from diploid blastulae, haploid blastulae, and from adult tissues: gill, pelvic fin, caudal fin, and viscera. Exact derivation conditions and culture media are described in detail in the appended Collodi *et al.*, 1992 manuscripts. Liver cell cultures were exposed to TCDD and the CYP1A response assessed by immunological methods using antiserum to trout CYP1A.

Gene transfer

Several approaches were taken; establishment of gene transfer in cultured cells, gene transfer by embryo microinjection, and transfer by electroporation. Transfer of plasmids such as pSV2neo into cultured cells was accomplished by traditional calcium phosphate-mediated transfection methods, followed by G418 selection (for details see appended manuscripts by Collodi *et al.*).

Transfer studies were conducted using a plasmid carrying the green fluorescent protein (GFP), as a marker gene which may be coupled to a transgene of interest and whose expression may be detected very simply and *in vivo* without sacrificing the animal. GFP, from the jellyfish *Aequorea victoria*, requires no supplemental co-factors or substrates to fluoresce green (max = 509nm) when bombarded with UV (λ_{max} = 395nm) or blue (λ_{min} = 470nm) light. The utility of the GFP expression system was first examined by electroporation into zebrafish cells in culture. The GFP-containing construct was pGFP-C1 (Clontech Laboratories, Inc., Palo Alto, CA), which was designed to be used to create fusion proteins to the C-terminus of GFP in the first reading frame under the control of the cytomegalovirus immediate early promoter. GFP-C1 DNA was amplified via growth overnight in a suitable host cell, and the DNA was recovered and purified using the Qiagen Plasmid Maxi Prep (Qiagen, Chatsworth, CA) according to the manufacturer's recommendations. The recovered DNA was then linearized via restriction at the *Dra* III site and purified with the GeneClean system (Bio101, Vista, CA). Three groups of 10^6 zebrafish liver epithelial cells at approximately 80% confluence were electroporated with 0, 20, or 20 μ g of linear PGFP-C1 DNA in the following manner. Cells were trypsinized to detach them from their flasks, centrifuged, washed, and then resuspended in 700 μ l Leibovitz's L-15 tissue culture medium. The cell suspension was then pipetted into 0.4cm gap electroporation cuvettes on ice and the linear GFP vector DNA was added. The ice incubation was continued for 10 minutes, then the electroporation pulses were delivered via a Gene Pulser apparatus (Bio-Rad, Hercules, CA). After electroporation, the cells were again iced for 10 minutes before being plated in 100mm plates in 10mls LDF media plus 5% fetal bovine serum (standard growth conditions). The media was changed 24 hours later. Selection with the aminoglycoside analog G418 was begun at 200 μ g/ml in the standard media on day 6 after electroporation, and incrementally increased up to 500 μ g/ml by day 20. The transfected cells were first visualized by epifluorescence microscopy on day 28 using a Zeiss inverted microscope and Zeiss FITC filter set under 200x magnification. Results were later photodocumented.

Transfection of zebrafish liver cells in culture with the mutation shuttle vector was examined via electroporation. Electroporation has proven to be a very successful methodology in our laboratory for these cells, resulting in transfection efficiencies far above those achieved with calcium phosphate co-precipitation methods. The lambda arms of the shuttle vector were engineered to contain a neomycin resistance cassette under the promotion of the SV40 long terminal repeat, and stable zebrafish cell transfectants were selected with neomycin.

RESULTS

Cloning, sequencing, expression of N-ras and p53

The enriched library approach allowed us to obtain a full-length zebrafish *ras* clone from the first plate (3×10^4 plaques) screened. This gene was termed Zras-B1. Northern blot hybridization analysis was carried out on total RNA isolated from synchronously-developing zebrafish embryos (maintained at 28°C) <1, 3, 6, 9, 12, 24, and 48 hours post-fertilization. A single transcript band of approximately 3.1 kb was identified using the Zras-B1 insert as probe. This transcript was most abundant in early embryos from the zygote stage through gastrulation (<1-6 hours), and thereafter gradually declined to barely detectable levels at 48 hours. The Zras-related mRNA detected in samples taken soon after fertilization is most likely maternally-derived. Northern hybridization analyses of the same RNA samples used as probes zL-myc, which was also present in maternally-derived mRNA, and zN-myc, which was not detectable prior to the onset of zygotic transcription. RT-PCR analysis indicated the temporal expression pattern of the zebrafish homologue of the murine *T* gene (*z-T*), with *z-T* first detectable as a very faint signal 3 hours post-fertilization but not prior to the midblastula transition.

The full length clone Z-p53m and the two partial overlapping clones Z-p53c and Z-p53f provided a complete zebrafish *p53* tumor suppressor cDNA sequence. Northern analysis employing the Z-p53c cDNA insert as a probe revealed the presence of a single 2.3 kb transcript in total RNA isolated from early zebrafish embryos. Relative levels of the zebrafish *p53*-related transcript were highest in newly-fertilized eggs, and gradually declined during the first 48 hours of embryonic development. Expression of the transcript within one hour following fertilization indicates that it is contributed, at least in part, maternally.

We had hoped at the outset of this work to be able not only to identify and sequence these genes, but to screen tumors for evidence of mutations. This work would have been relatively straight forward had zebrafish developed large, easily excised tumors readily locatable with the naked eye. This did not turn out to be the case, however. Tumors to date have been detectable only through staining of histological sections. Histological fixatives other than Bouin's solution will be needed to avoid DNA fragmentation, so that the DNA of these microscopic tumors can be PCR amplified for mutational screening.

Zebrafish cell lines

Several cell lines were successfully established, and shown to be karyotypically diploid. The diploid blastula line ZEM has been maintained for over 100 passages now and is a well established cell line. An important feature was the need for insulin as well as a growth factor that we discovered from trout serum that supports growth and doubling of these cells. We examined the response of these cells in culture to exposure to several classic Ah receptor agonists. Cultured liver cells were treated with classic inducers of phase 1 xenobiotic metabolizing enzymes. Immunoblots assessed the response of homologues of trout P450s LMC1-LMC5, and of CYP1A which is known to be regulated by Ah receptor agonists in mammals. Although no proteins orthologous to the LMC P450s were detected, a CYP1A

orthologue was strongly induced by TCDD treatment. This protein carried ethoxyresorufin-O-deethylase activity, which is typical of CYP1A enzymes, and metabolized the carcinogen DMBA to its 3,4-diol. Cultured cells did not show induction when treated with another classic Ah agonist β -naphthoflavone.

Gene transfer

Plasmid pSV2neo was found to confer G418 resistance to several zebrafish cell lines through stable genomic integration at frequencies comparable to mammalian cells. We compared transient expression of plasmids with bacterial B-galactosidase under control of SV40 early promoter or CMV immediate early promoter. Good expression with either promoter was dependent on the presence of a splice signal. We further showed that cultured blastula-derived cells survive injection into fertilized embryos. Recently we have evidence that injected cells carrying marker genes contribute to formation of chimeric zebrafish (unpublished), although germ-line transmission has not been established for the marker genes.

In the GFP studies, many colonies of stable G418-resistant clones arose under selection, only 1-10% of these were observed to express GFP. Most GFP-expressing cells were isolated with only partial evidence for clonal expansion apparent. GFP fluorescence was often quite bright, but was observed to be rather variable among the GFP-expressing cells. Similar results have been reported by other researchers. Passage of the cells did not appear to increase clonal expansion of the GFP transfected cells. In this experiment, we did not attempt to assess the level of transient GFP expression. The fluorescing cells observed are almost certainly stably transformed with the GFP sequence. The next studies will be to electroporate early zebrafish embryos with the linear GFP vector to further assess the utility of this marker gene. For the mutational cassette experiments, initial results indicate that the lambda cassette can be successfully electroporated into a variety of cell lines, and that G418 selection is a viable approach with zebrafish cells. However, G418 selection seems unable to identify zebrafish cells expressing this marker as it has been incorporated into the lambda mutation cassette. This suggests some form of selectable marker silencing has occurred, and current experiments are investigating a solution to this problem.

DISCUSSION

These studies provide the first description of a *ras* and a *p53* gene in zebrafish. These sequences are expected to be of great use due to the expanding use of this model for developmental studies. Overall, Zras-B1 is 91, 84, and 85% homologous to human N-, Ha-, and Ki-ras p21, respectively. Thus, by simple amino acid sequence comparison zebrafish Zras-B1 shows closest identity to mammalian N-ras and is tentatively identified as an N-ras. We stress, however, the precise differences that confer biologically significant properties unique to N-, Ha-, and Ki-ras p21 are not known, and it is thus not possible to conclude that the protein encoded by Zras-B1 functions in zebrafish as an N-ras protein.

Comparison of the zebrafish *p53* deduced protein sequence with that of trout and human (Figure 4) reveals that 63% and 48% of the residues, respectively, are identical. Not

surprisingly, the alignment reveals quite striking sequence homology in conserved domains I through V between these three species, as well as across all 14 species compared. The most significant divergence between the zebrafish p53 sequence and other species, including trout, is evident in the amino-terminal region of the protein, which also encompasses conserved domain I. Divergence is again more apparent in the carboxyl-terminal region.

These experiments also demonstrated the expression of an *N-ras* proto-oncogene and a *p53* tumor suppressor gene early in embryonic development. Our tumor studies have independently indicated that zebrafish appear most vulnerable to tumor initiation when treated at early life stages with carcinogens. One possible explanation is that the expression of these two genes, which are critical targets for mutational inactivation in most other animal and human cancers, are especially vulnerable to mutagenesis in the zebrafish at this life stage.

Our attempts to produce zebrafish cell lines for further study were very successful, and these are proving useful for studies of gene regulation and xenobiotic metabolism. Moreover, the most promising approach in our hands for production of transgenics is the transfer of genes into diploid embryo cell lines, selection for expression, and injection of these cells into fertilized embryos. We have been able, for example, to demonstrate the participation of cells from normal zebrafish in embryonic development when injected into albino recipient embryos. This was readily visualized as albino hatchlings with mosaic distribution of pigmented cells (Barnes, unpublished results). Our experiments involving direct microinjection or electroporation of DNA into fertilized embryos have been less promising to date. Though we have observed a few hatchlings expressing *Lac* transgenes as mosaics, the detection requires sacrifice and prohibits further mating experiments. The GFP approach is just under way, but may allow a ready identification of transgenics to serve as founders. The use of PCR methods on fin clip DNA works in our hands, but is very laborious and does not guarantee that the transgene is expressed.

CONCLUSIONS

While we did successfully characterize the metabolism and hepatic DNA-adduction of AFB₁ in zebrafish, we have yet to characterize the metabolism of additional carcinogens such as methylazoxymethanol acetate, MAM-Ac, DEN and DMBA. Early in our studies a drawback of zebrafish became quite apparent, namely that the biology of this species greatly impedes biochemical studies. The zebrafish liver is small, diffuse, and difficult to remove, and large numbers of livers must be pooled to obtain enough tissue with which to work. In addition, because it is virtually impossible to remove the liver intact, proteases are apparently released during sampling, which often can result in the rapid loss of protein biologic activities. We also experienced difficulty in i.p. injecting small zebrafish without serious injury to the fish, yet fish grown to larger size become increasingly susceptible to oodinium infection. Larger zebrafish also require a substantial amount of tank space, yet another restriction we faced. Consequently, the number of fish needed for these experiments was often the limiting factor.

Our overall recommendation is that for metabolic studies, one may make greater progress using molecular methods to clone and express zebrafish P450s and other genes in heterologous expression systems, as we are currently doing for trout genes of interest. Though this approach is technically more elaborate, recent advances make the approach more accessible to the average facility. Once a panel of cloned and expressed genes is established, the study of many xenobiotics can be accomplished rapidly and in parallel.

Our *ras* and *p53* studies have paved the way for an understanding of the genetic events critical to cancer development in the zebrafish. With an appropriate histological staining method, it should be quite feasible to excise malignant cells from a fixed slide of tumor specimens to screen for mutations in *N-ras* and/or *p53*. Based on work in trout, we fully expect to find *ras* mutations; these could be useful in characterizing the potential mutagenic carcinogens to which fish in *in situ* field monitoring may have been exposed. The *p53* sequence may be of greater use to developmental biologists, for example in the construction of *p53* knockout zebrafish. To date this tumor suppressor gene has been found to be mutated far less frequently in animals than in human cancers.

The ability to establish replicating cell cultures that maintain diploid karyotype, responsiveness to enzyme inducers, capability to metabolize procarcinogens, and ability to express transgenes represents a significant advance in the potential utility of the zebrafish model. Though we did not establish germline transmission of expressed foreign genes within the time period of this contract, recent advances elsewhere in the design of fish promoters that retain expression (e.g. Wang *et al.*, 1995) indicate that this should be possible. The mutational cassette currently under investigation may provide transgenic zebrafish or other aquarium fish with high sensitivity to detect mutagenic hazard in waters to which they might be exposed.

Our goals for this project were to understand in detail the carcinogen metabolism capabilities of zebrafish and the mechanistic support this knowledge would have given to a

carcinogenesis model. Additional goals to document oncogene involvement in zebrafish carcinogenesis and to construct exquisitely sensitive transgenic fish for environmental monitoring, have met with varied success due in large part to unforeseen obstacles inherent in small fish research. Regardless of our successes or failures with these objectives, the questions that ultimately would determine the usefulness of zebrafish as a model for environmental monitoring of carcinogens were: 1) How does this species respond to known carcinogens?, and 2) Is its biology adaptable to the rigors of environmental monitoring?

To answer these questions, we have looked at six different carcinogens, and four routes of exposure. We realize this is not a broad basis on which to evaluate a model, but these studies do provide us with sufficient evidence to form a hypothesis. While it is probably inaccurate to compare sensitivities based on dose levels in diets or in exposure waters, at this time these are the only comparisons we have to work with. On this basis, zebrafish are definitely more resistant to neoplasia than are rainbow trout (the most established fish model) (Bailey *et al.*, 1996), by as much as three orders of magnitude with respect to dietary aflatoxin B₁ (AFB₁), and to lesser degrees with other carcinogens. This was not surprising since comparisons between rainbow trout and other aquarium fishes have usually shown the trout to be more sensitive on an administered dose basis. Although space and time constraints prevented more comparative studies between zebrafish and medaka, the one comparison we made using dietary methylazoxymethanol acetate (MAM-Ac), revealed similar sensitivities in these two species. Thus, with the possible exception of AFB₁, to which zebrafish appear to be particularly resistant, the sensitivity of zebrafish to carcinogens may be relatively similar to other small aquarium fishes, and would not disqualify them as a potential model.

These experiments have also revealed some important features about the suitability of various exposure routes. For small fish, the involuntary water bath exposures are preferable to dietary exposure for several reasons. First, the protocol that we have used for starting newly hatched zebrafish on food requires the use of either live food or food that comes in very small particle size. Neither of these food sources are amenable to accurate incorporation of a toxin or carcinogen. Thus and secondly, the extremely small size of the young fish make it almost mandatory that dietary exposures be delayed until the fish achieve some minimal size that will allow acceptance of a minimal dietary particle size of a diet into which the toxin can be incorporated, whereas with bath exposures, size of the fish is immaterial. Third, we have found that the delay to increase the fish size required about two months for zebrafish, a delay which may lower their sensitivity due to age, based on information gained from rainbow trout experiments (unpublished results from our laboratory). Conversely, bath exposures give the scientist complete flexibility on the timing of the exposure. Fourth, this delay in time also results in the zebrafish being close to or already at sexual maturity, a change that can introduce another variable into the neoplasia formula. And fifth, we have found that zebrafish tend to eat over an extended period of time rather than the aggressive, all-at-once feeding behavior of rainbow trout. This tendency makes it more difficult to know how much diet to feed, and some of it may lay on the bottom for extended periods of time, and either not being eaten at all or leach an unknown amount of toxin into the water.

The final, most obvious, most important, and yet completely overlooked characteristic of zebrafish that determines their suitability and versatility as a model for field monitoring of carcinogenic hazards, is their lack of tolerance for the range of temperatures that would be encountered in such monitoring experiments. The extensive literature review on the biology and use of zebrafish by Laale (1977), did not discuss temperature tolerance as a limiting factor, and as a result we failed to realize the importance of this parameter. In brief, we know from limited experiments that zebrafish cannot tolerate temperatures less than or equal to 10°C, since these temperatures have an almost immediate anesthetic, immobilizing, effect on the fish. Temperatures between 10 and 15°C greatly reduce the fish's activity within the first minute, and although we do not know what the long-term effects would be, we assume the fish would be under severe stress, would not feed normally, and their ability to withstand chemical and/or biological (infectious) assault would be greatly compromised. Thus, based on this very basic requirement, zebrafish are unsuitable as a field monitoring species in temperate or colder climates. Their usefulness is restricted to laboratory and/or tropical or subtropical field studies only.

Since we have not discovered any compelling advantages of zebrafish over some of the other models (e.g., the guppy) currently being used in support of the Japanese medaka, we do not recommend thrusting the zebrafish into this role.

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LIST OF PERSONNEL

Arbogast, Daniel
Barnhill, Jean
Cheng, Ronshan
Cleveland, Sheila
Collodi, Paul
Doolin, Pearl
Dutchuk, Mike
Ellis, Theresa
Loveland, Pat
Macdonald, Chance
Mathews, Catherine
Miller, Tomas
O'Neal-Tragen, Patricia
Owston, Connie
Reddy, Ashok
Troxel, Claudia
Tsai, Hsi-Wen

APPENDICES

**THE CARCINOGENIC RESPONSE OF ZEBRAFISH (*BRACHYDANIO RERIO*)
TO N-NITROSODIETHYLAMINE AND N-NITROSODIMETHYLAMINE
BY THREE EXPOSURE ROUTES**

Hsi-Wen Tsai², Jan Spitsbergen², Dan Arbogast²,
Ashok Reddy² and Jerry Hendricks^{1,2}

¹Marine/Freshwater Biomedical Sciences Center

²Department of Food Science and Technology

Oregon State University, Corvallis, OR

ABSTRACT

Zebrafish (*Brachydanio rerio*) were exposed to N-nitrosodiethylamine (DEN) and N-nitrosodimethylamine (DMN) by dietary or short-term embryo or fry water-bath exposures. Dietary doses of 500, 1,000, or 2,000 ppm of each nitrosamine for 12 wk failed to initiate any neoplasms 26 wk after the start of exposure. Sixteen-day post-hatch fry were exposed to aqueous concentrations of 500, 1,000, 1,500, or 2,000 ppm DEN and 250, 500, 1,000, 2,000, or 2,500 ppm DMN for 24 hr and terminated 12 mo later. Both carcinogens caused dose-responsive neoplasia in the liver. Embryos were exposed to DEN, only, at doses of 1,000, 2,000, or 3,000 ppm for 24 hr. All doses produced a low incidence of hepatic neoplasms. Several other neoplasms, mostly of mesenchymal origin, were also diagnosed in this group. In general, zebrafish were not as responsive as anticipated with respect to hepatocarcinogenesis, but they did respond with several infrequently observed neoplasms in other organs.

INTRODUCTION

Over the last three decades, fish have been used in a number of ways to increase our understanding of the biology of neoplasia. These include: 1) as low-cost, whole-animal carcinogenesis models (Sinnhuber *et al.*, 1977; Hawkins *et al.*, 1985), 2) as comparative vertebrates for mechanistic research (Dashwood *et al.*, 1988; Bailey *et al.*, 1996), and 3) as carcinogen-indicator organisms in the natural environment (Walker *et al.*, 1985; Hawkins *et al.*, 1988a). Rainbow trout, *Oncorhynchus mykiss*, have played a dominant role in these research efforts (Sinnhuber *et al.*, 1977; Hendricks, 1994; Bailey *et al.*, 1996), but long generation time, strict water quality requirements, and relatively large space needs have prevented widespread use of this model animal. As a result, small aquarium species, which have short generation times and less demanding water, space, and cost requirements, were logical alternatives to the trout as useful aquatic models (Matsushima and Sugimura, 1976; Hawkins *et al.*, 1988b).

The first small fish carcinogenesis experiment reported in the literature used the popular aquarium species, the zebrafish (*Brachydanio rerio*), a cyprinid fish native to India. The fish were exposed to N-nitrosodiethylamine (DEN) continuously in the water, and developed both hepatic and biliary cell neoplasms (Stanton, 1965). The reasons why zebrafish did not subsequently develop into a widely used small fish model for carcinogenesis research are not known. Only one other carcinogenesis study using zebrafish, also with nitrosamines, is present in the literature (Khudoley, 1984). Rather than building on Stanton's early use of the zebrafish, however,

various groups began using several other species for cancer research. Thus, the guppy (*Poecilia reticulata*) (Khudoley, 1972; Khudoley, 1984; Sato *et al.*, 1973; Parland and Baumann, 1985; Fournie *et al.*, 1987, Hawkins *et al.*, 1989), Japanese medaka (*Oryzias latipes*) (Ishikawa *et al.*, 1975; Aoki and Matsudaira, 1977; Egami *et al.*, 1981; Hatanaka *et al.*, 1982; Hinton *et al.*, 1984; Hawkins *et al.*, 1986; Bunton, 1989; Bunton and Wolfe, 1996a; 1996b) *Poeciliopsis* sp. (Schultz and Schultz, 1982a; 1982b; 1985; 1988), and *Rivulus marmoratus* (Koenig and Chasar, 1984; Grizzle and Thiyagarajah, 1988) have been used to varying degrees over the past fifteen years.

Zebrafish have been used extensively for other areas of research, chiefly developmental biology (Laale, 1977; Stainer and Fishman, 1992; Eisen and Weston, 1993; Kimmel and Kane, 1993) and neurobiology (Van Asselt *et al.*, 1991; Chitnis *et al.*, 1992; Bernhardt, *et al.*, 1992). Based on the wealth of background information known about this species from the studies in these other areas, and the original work by Stanton (1965), this species seemed to warrant a systematic appraisal of its value as a model for carcinogenesis. The objectives of this report were to assess the dose-response of zebrafish to two extensively studied nitrosamine carcinogens, N-nitrosodiethylamine (DEN) and N-nitrosodimethylamine (DMN), by three exposure routes, and to histologically describe the induced lesions.

MATERIALS AND METHODS

Chemicals

N-nitrosodiethylamine (DEN) was purchased from Fluka Chemical Corporation (Ronkonkoma, NY). N-nitrosodimethylamine (DMN) was purchased from Sigma Chemical Company (St. Louis, MO).

Animals

Zebrafish were initially obtained from 5-D Tropical Fish (Plant City, FL). Thereafter, they were spawned and reared at the Food Toxicology and Nutrition Laboratory (FTNL), Oregon State University.

Culture Conditions

Fish were reared in the well water normally used for salmonid culture at the FTNL, but this water had to be treated in the following ways. It was run through a column of plastic coils to remove excess nitrogen gas, buffered to a pH of 7.0-7.2 with a phosphate buffering system, and heated to a temperature between 24 and 26°C. Experimental groups of fish were held in 110 L tanks. Ten times weekly, 4 L of new water was added to these tanks and 4 L was removed through an overflow system. A 14-hr light/10-hr dark photoperiod cycle was used and each tank was equipped with an airstone for aeration.

Fish were fed according to the following schedule. About five days after hatching, zebrafish larvae were fed Microfeast Plus larval diet (Provesta Corp., Bartlesville, OK) 3-5 times daily. After about two wk, they were started on brine

shrimp, and slowly (over 3-5 days) weaned off Microfeast. They received brine shrimp only until about 6 wk old, and from that time on they were fed Oregon Test Diet (OTD) (Lee *et al.*, 1991) twice daily and brine shrimp once daily. This diet regimen was continued throughout the experiments except in the dietary exposure groups, described below.

Diets

Purified casein diet (PC diet), which was developed at the University of California, Davis, for use with Japanese medaka (*Oryzias latipes*) (DeKoven, *et al.*, 1992), was used as the basic diet in our feeding experiments. However, for dietary carcinogen exposures, we preferred to have the diet in a gelatinized rather than powdered form. Thus we added 2% gelatin to the basic PC diet formulation, and made a moist diet by thoroughly mixing 65% hot water ($\approx 55^{\circ}\text{C}$) with 35% dry mix and cooling in a refrigerator. In this form the diet retained its integrity in the water longer, resulting in more complete consumption of the administered amount.

Fish used in the fry and embryo exposure experiments were fed OTD, which has been developed at OSU for use with rainbow trout, and brine shrimp.

Dietary Exposure

DEN or DMN, at doses of 500, 1,000, or 2,000 mg/kg (ppm) diet was added to the hot water before combining with the other ingredients of the modified PC diet, and mixing as described above. The diets were fed by expressing the diet through a fine meshed screen mounted on a caulking gun and cutting with a spatula.

Duplicate groups of 100, 8-wk-old zebrafish, selected randomly with regard to sex, were placed in 110 L tanks and fed the control, 500, 1,000, or 2,000 ppm diets for 12 wk. Fish were then maintained on the modified PC diet for an additional 14 wk until the experiment was terminated.

Fry Bath Exposure

Single groups of 120 zebrafish fry, 16 days post-hatch, were exposed to buffered water solutions of DEN (no solvent carrier was used) at concentrations of 500, 1,000, 1,500, and 2,000 ppm for 24 hr under static conditions. Control fish were held under similar conditions but without the DEN. After exposure, each group of fish was placed in a 110 L glass tank and fed OTD and brine shrimp for 12 mo. At termination, all fish were killed and fixed for histopathological diagnosis. The procedures for DMN exposure were the same, but the DMN doses used were 250, 500, 1,000, 2,000, and 2,500 ppm. Abnormally high mortalities occurred from time to time during the experiment, resulting from a parasitic outbreak of *Oodinium sp.* Acriflavine, at a dose of 5.4 ppm, was added to the water as needed to control this problem.

Embryo Bath Exposure

Duplicate groups of 150 zebrafish embryos, 60 hr post-fertilization, were placed in static, buffered water solutions of DEN at 1,000, 2,000, and 3,000 ppm for 24 hr. After treatment the embryos were rinsed in clean water and placed in 1.5 L beakers until hatching at 96 hr. The resulting fry were started on feed and kept in the 1.5 L beakers for one mo before transferring to 110 L glass tanks. They were fed

OTD for 12 mo, killed in tricaine methane-sulfonate (MS 222) anesthetic and fixed for histopathological diagnosis. No embryos were exposed to DMN.

Tissue Preparation and Tumor Detection

At necropsy, all fish were anesthetized in MS-222, weighed and measured. Scales, fins, and the caudal peduncle were removed, the body cavity opened ventrally, and the swim bladder punctured prior to fixation in Bouin's solution. After 24 hr fixation, the fish were dehydrated, infiltrated, and embedded in paraffin on their side so that sagittal sections could be cut from the left side. Three 5-6 μm sections were kept from each fish (one at mid-eye level, one just past the eye, and one in the midline), mounted on a single glass slide, and stained with hematoxylin and eosin. Organ accountability was good using this procedure. Tumor detection was based solely on observations on the three saved sections.

Statistics

Tumor incidence data were analyzed by logistic regression with categorical and/or continuous predictors in the Genmod procedure of SAS (SAS, 1996). For experiments with replicate tanks, there was no evidence of overdispersion ($p > 0.5$, all lack of fit tests) and residuals appeared consistent with the binomial error model. For the fry water bath exposure to DEN, the usual large-sample Chi-square p-value was quite close to 0.05, and for that case, an exact permutation p-value is also given as generated by StatXact version 3.1 (Mehta and Patel, 1995).

RESULTS

Dietary Exposure

There were no differences in growth or behavior of the various experimental and control groups for both DEN and DMN during the 6-mo trial period. All ate equally well and responded normally to external stimuli. Mortalities were low except for unexplained losses in three tanks. No pathologic changes in the liver or other organs were detected by histologic examination after 26 wk (Tables 2.1 and 2.2).

Table 2.1. Carcinogenic response of zebrafish to 12-wk dietary DEN exposure

Lot	DEN dose ppm	Mortality %	Neoplastic response ^a	
			Inc.	%
1	Control	3	0/97	0
2	Control	2	0/98	0
1	500	7	0/93	0
2	500	15 ^b	0/85	0
1	1000	20 ^b	0/80	0
2	1000	2	0/98	0
1	2000	6	0/94	0
2	2000	0	0/100	0

^aFish necropsied 6 mo after start of carcinogen exposure

^bUnexplained loss of fish

Table 2.2. Carcinogenic response of zebrafish to 12-wk dietary DMN exposure

Lot	DMN dose ppm	Mortality %	Neoplastic response ^a	
			Inc.	%
1	Control	20 ^b	0/80	0
2	Control	1	0/99	0
1	500	5	0/95	0
2	500	0	0/100	0
1	1000	0	0/100	0
2	1000	0	0/100	0
1	2000	0	0/100	0
2	2000	0	0/100	0

^aFish necropsied 6 mo after start of carcinogen exposure

^bUnexplained loss of fish

Fry Bath Exposure

Cumulative mortality in the DEN experiment was higher than desirable and partially the result of an *Oodinium sp.* parasitic outbreak. However, there was also mortality associated with DEN exposure, especially at the highest dose (Table 2.3). For fish that survived, there was evidence of differences in neoplastic response between the four lots receiving DEN ($p=0.0509$, standard Chi-square approximation, 3 df) and ($p=0.0485$, exact permutation, 3 df). Over the four DEN doses (500 to 2000 ppm) there is a significant linear dose response ($p<0.0134$, logistic regression, 1 df) with no evidence of lack of fit to linear ($p>0.5$, 2df).

Table 2.3. Carcinogenic response of 14 day post-hatch zebrafish fry exposed to static water solutions of DEN for 24 hr, and terminated 12 mo later

Lot	DEN dose ppm	Mortality %	Neoplastic response	
			Inc. ^a	% ^b
1	Control	32	0/82	0
2	500	60	5/48	10
3	1000	52	7/58	12
4	1500	50	14/61	23
5	2000	98	2/3	67

^aNo. of fish with at least one neoplasm/total No. of fish

^bOver the four DEN doses, there is a significant linear dose response ($p<0.0134$, 1df, logistic regression)

Mortalities occurred in the DMN experiment as well but were not dose responsive (Table 2.4). Among those that survived, there were highly significant differences in neoplastic response between lots ($p < 0.0001$, 5 df). There was increasing response with DMN dose until the highest dose which results in strong evidence of curvilinearity in the DMN dose response ($p < 0.0001$, 1 df quadratic term) and no evidence of lack of fit to quadratic ($p > 0.50$, 1 df).

Table 2.4. Carcinogenic response of 14-day post-hatch zebrafish fry exposed to static water solutions of DMN, and terminated 12 mo later

Lot	DMN dose ppm	Mortality %	Neoplastic response Inc. ^a	% ^b
1	Control	1	0/119	0
2	250	17	0/100	0
3	500	30	1/84	1
4	1000	17	12/100	12
5	1500	22	25/94	27
6	2000	50	26/60	43
7	2500	33	31/81	38

^aNo. of fish with at least one neoplasm/total No. of fish

^bAmong survivors, there are highly significant differences in neoplastic response between tanks ($p < 0.0001$, 5df).

Histopathologic lesions were limited primarily to the liver. To understand the changes that occurred in the liver due to carcinogen action, it is essential to know the organization of the normal zebrafish liver. It consists of hepatocytes arranged in tubules, sinusoids that surround the tubules, and infrequent portal tracts containing bile ducts, hepatic arteries, and connective tissue (Fig. 2.1). Hepatocyte nuclei are uniform in size, circular to slightly oval, and have a large, distinct nucleolus. Longitudinally, tubules are two cells wide between adjacent sinusoids, while cross sections appear as tubules with 5-8 hepatocytes surrounding an inconspicuous central

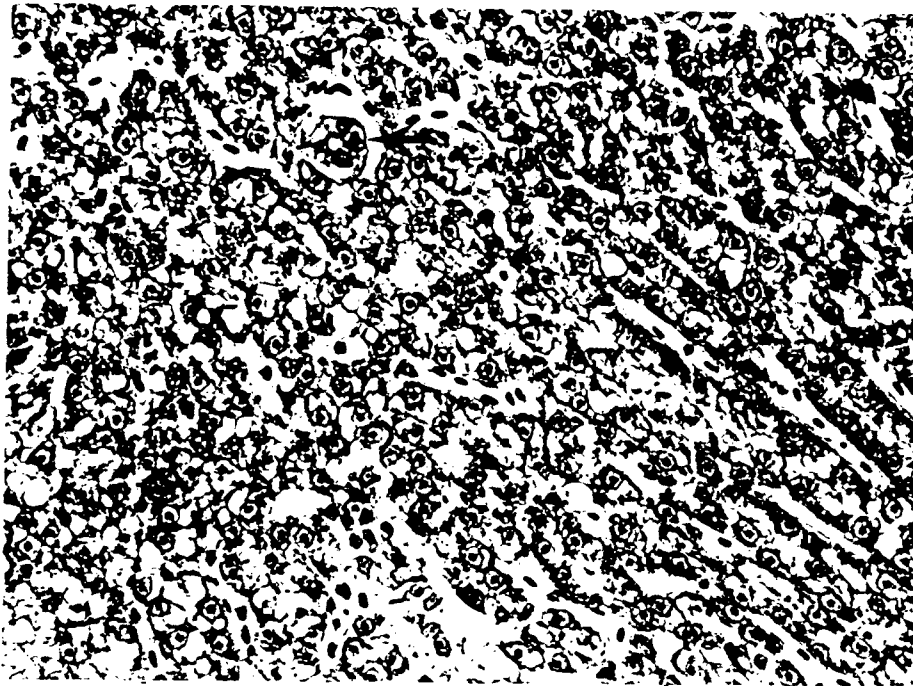


Fig. 2.1. Normal liver from control zebrafish. Note hepatic tubules, two cells wide in longitudinal section, uniform nuclear size with prominent nucleolus, the moderate level of glycogen vacuolization, and the small biliary duct (arrow). H&E, X544.

bile canaliculus. All degrees of oblique sectioning through the tortuous hepatic tubules give rise to the remaining appearance of the hepatic parenchyma. The numbers and types of lesions are presented in Tables 2.5 and 2.6.

Table 2.5. Numbers of neoplastic and associated lesions of zebrafish taken 1 yr after a 24-hr fry-bath exposure to DEN

Lesion types	Total No. of neoplasms observed at each dose of DEN (ppm)				
	0	500	1000	1500	2000
Non-neoplastic lesions					
CT*	0/82	1/48	3/58	11/61	0/3
BH	0/82	3/48	13/58	14/61	1/3
Foci of altered cells					
BF	0/82	2/48	2/58	3/61	0/3
EF	0/82	2/48	8/58	5/61	0/3
Neoplasms					
HCA	0/82	2/48	4/58	6/61	1/3
HCC	0/82	0/48	1/58	3/61	0/3
CCA	0/82	1/48	1/58	0/61	0/3
CCC	0/82	3/48	4/58	8/61	1/3

*Abbreviations used: CT - cytotoxicity, BH - biliary hyperplasia, BF - basophilic foci, EF - eosinophilic foci, HCA - hepatocellular adenoma, HCC - hepatocellular carcinoma, CCA - cholangiocellular adenoma, CCC - cholangiocellular carcinoma

Table 2.6. Numbers of neoplastic and associated lesions of zebrafish taken 1 yr after a 24-hr fry-bath exposure to DMN

Lesion types	Total No. of tumors observed at each dose of DMN (ppm)						
	0	250	500	1000	1500	2000	2500
Non-neoplastic lesions							
CT*	0/119	0/100	1/84	11/100	6/94	3/60	3/81
BH	0/119	0/100	1/84	5/100	8/94	2/60	0/81
Liver lesions - foci of altered cells							
BF	0/119	0/100	0/84	1/100	1/94	3/60	1/81
EF	0/119	0/100	0/84	2/100	3/94	1/60	3/81
CF	0/119	0/100	0/84	0/100	2/94	1/60	3/81
Liver neoplasms							
HCA	0/119	0/100	0/84	1/100	6/94	5/60	4/81
HCC	0/119	0/100	0/84	0/100	0/94	1/60	2/81
CCA	0/119	0/100	0/84	0/100	5/94	8/60	8/81
CCC	0/119	0/100	1/84	11/100	15/94	16/60	15/81
HS	0/119	0/100	0/84	0/100	0/94	0/90	1/81
Intestinal neoplasms							
LS	0/119	0/100	0/84	0/100	0/94	2/90	5/81

*Abbreviations used: CT - cytotoxicity, BH - biliary hyperplasia, BF - basophilic foci, EF - eosinophilic foci, CF - clear cell foci, HCA - hepatocellular adenoma, HCC - hepatocellular carcinoma, CA - cholangiocellular adenoma, CCC - cholangiocellular carcinoma, HS - hemangiosarcoma, LS - leiomyosarcoma

Focal cytotoxic changes to the hepatic parenchyma occurred at all dose levels of both DEN and DMN and was dose related. Irregularity in nuclear size and shape, cell swelling and the resulting loss of normal tubular and sinusoidal architecture were the primary signs of cytotoxicity observed (Fig. 2.2). In general these changes were not severe enough to be life threatening. Biliary hyperplasia also occurred regularly in the livers of fish exposed to both DEN and DMN. This ductal proliferation along biliary tracts was in marked contrast to the infrequent occurrence of biliary ducts in the normal zebrafish liver.

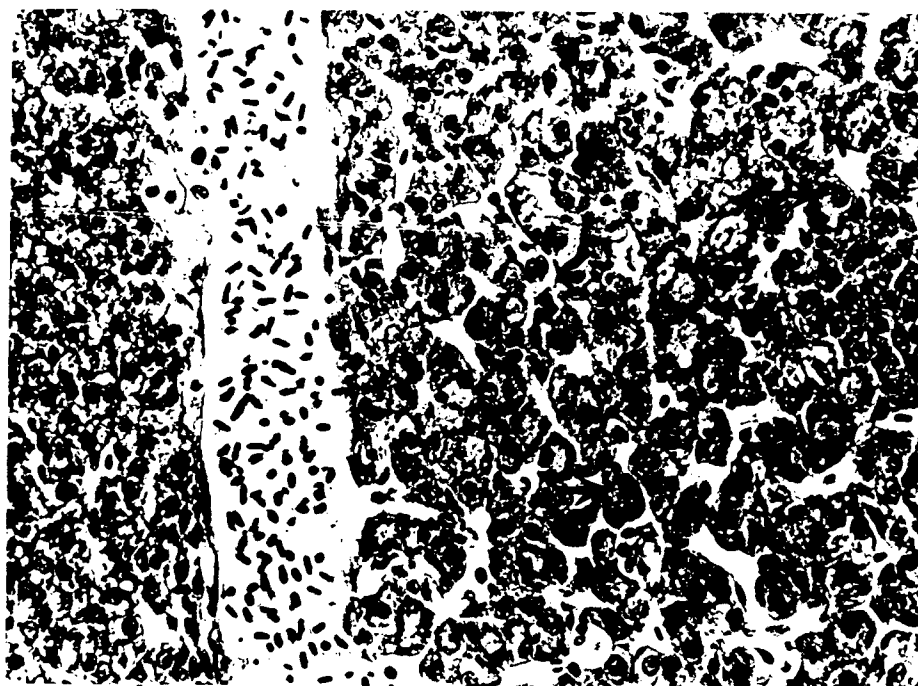


Fig. 2.2. An area of cytotoxicity in the liver of a zebrafish exposed to 1500 ppm DEN as a fry. Note reduced glycogen vacuolization, cell swelling and loss of nuclear uniformity (arrows). H&E, X544.

Foci of cellular alteration were observed with exposure to both nitrosamines. We are not sure if these lesions progress to become neoplasms, so we are not including them in that category. Eosinophilic foci (Fig. 2.3) were the most common in both groups, with fewer numbers of basophilic foci (Fig. 2.4) and some clear cell foci (Fig. 2.5) in the DMN group only. Eosinophilic foci had dense, granular, eosinophilic cytoplasm with little or no vacuolization. Basophilic foci were distinctly basophilic in staining and had denser, less vacuolated cytoplasm. Clear foci consisted of cells with clear cytoplasmic areas surrounding either centric or eccentric nuclei.

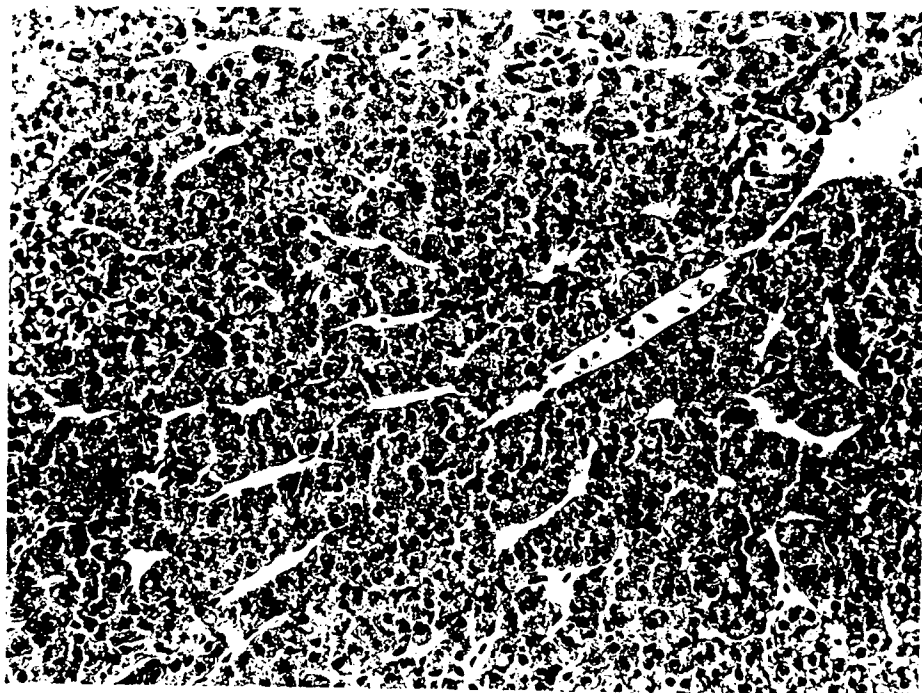


Fig. 2.3. An eosinophilic focus in the liver of a zebrafish exposed to 1000 ppm DEN by fry exposure. Cells occupy normal hepatic tubules, but are distinctly eosinophilic in staining, enlarged, and have more irregularity in nuclear size, shape, and staining and less glycogen than the surrounding normal hepatocytes. H&E, X340.

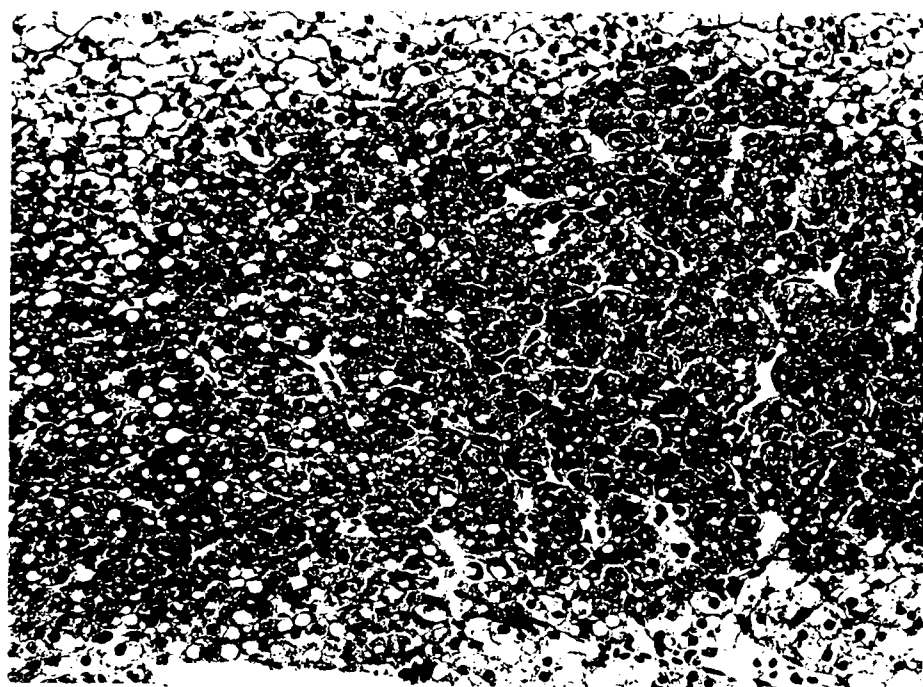


Fig. 2.4. A basophilic focus from the liver of a zebrafish exposed to 1500 ppm DEN by fry bath. The cells are swollen, with nuclear irregularity, less glycogen, and distinct basophilia. In nearly all cases, however, they still occupy normal hepatic tubules. H&E, X340.

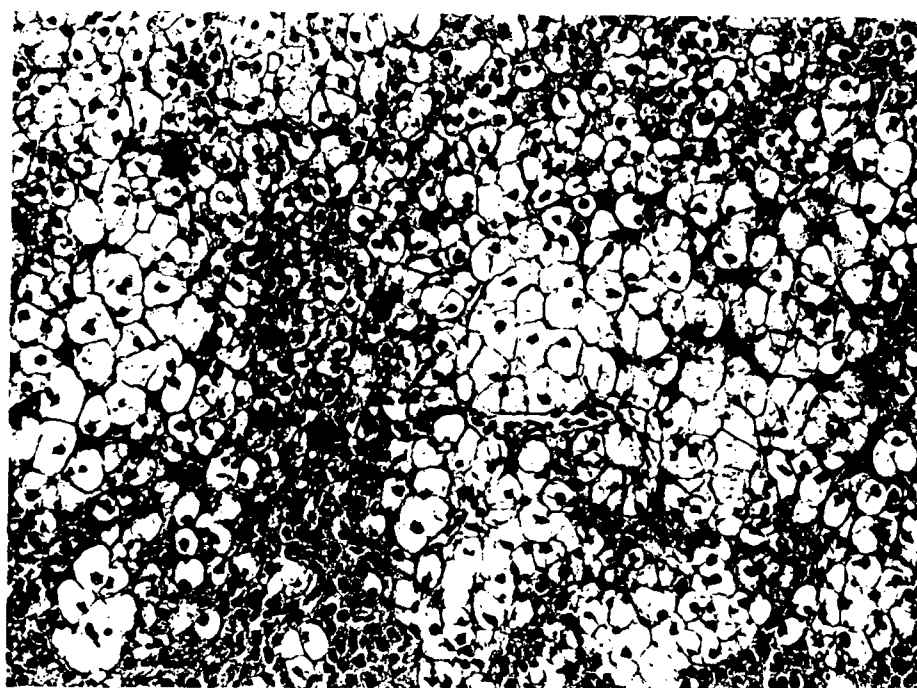


Fig. 2.5. A focus of clear cells in the liver of a zebrafish exposed to 2500 ppm DMN as a fry. Cells are enlarged, engorged with glycogen, and have either centric or eccentric, condensed nuclei. Normal tubular pattern is obliterated due to extreme swelling. H&E, X 340.

Hepatocellular adenomas were more numerous than hepatocellular carcinomas in both groups. They were expansive (compressive), nodular growths of either eosinophilic (most common) or basophilic hepatocytes that still retained their normal tubular arrangement. Mitotic figures were rare, and cytoplasmic and nuclear features were similar to those seen in foci of altered hepatocytes (Fig. 2.6).

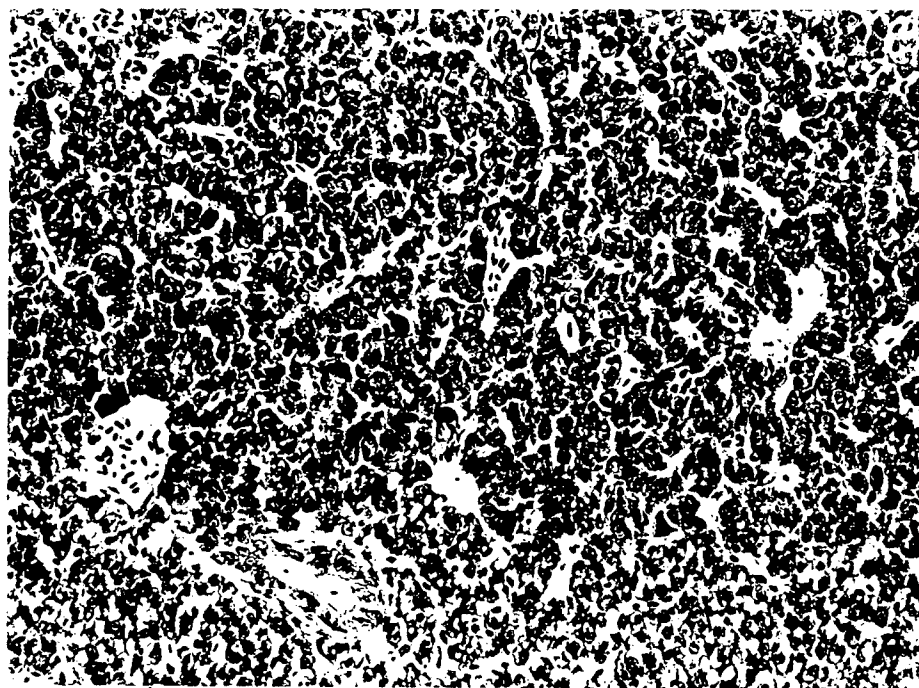


Fig. 2.6. A portion of a basophilic hepatocellular adenoma in a zebrafish exposed to 1500 ppm DMN as a fry bath. Cellular characteristics are similar to those of basophilic foci, but the lesion is larger and causes some compression of surrounding tissue. H&E, X340.

Hepatocellular carcinomas were rare with both carcinogens. They consisted of large nodular growths, of either eosinophilic or basophilic cells. The definitive characteristic of these tumors is that the cells no longer occupy normal hepatic tubules, but rather the hepatic tubules become engorged with neoplastic hepatocytes, presenting a condition of multiple cells between adjacent sinusoids (Fig. 2.7). The cells tend to be more varied in nuclear and cytoplasmic features than adenoma cells, and display some mitotic figures. Frequent apoptotic bodies were also observed in carcinomas.

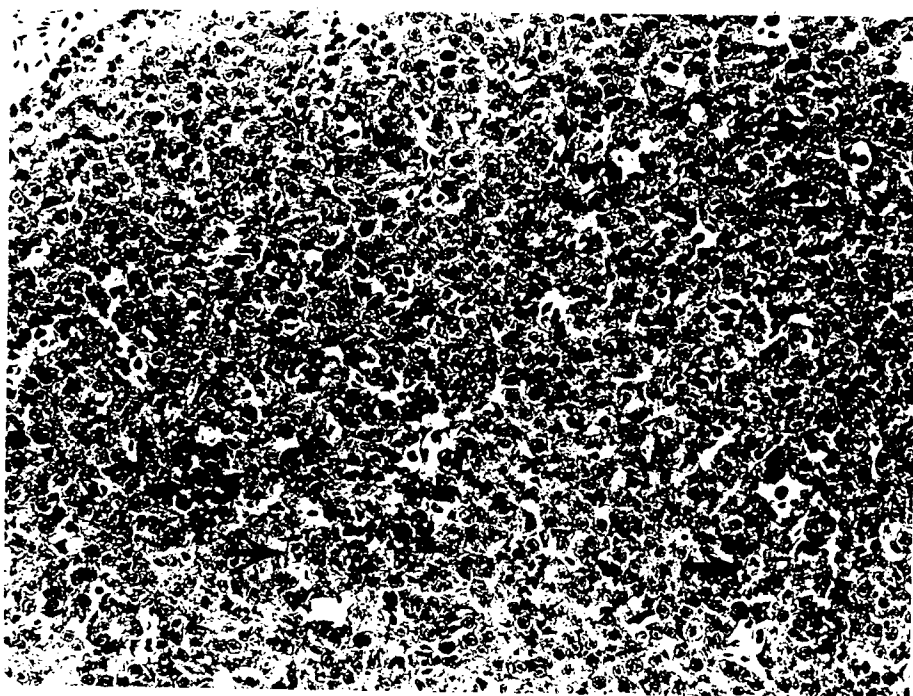


Fig. 2.7. A portion of a large hepatocellular carcinoma in a zebrafish exposed to 1000 ppm DEN by fry bath. Numerous cells occur between adjacent sinusoids, mitotic figures are common (arrows), as are apoptotic cells. H&E, X340.

Cholangiocellular adenomas were a common neoplasm especially in the DMN-exposed groups. These lesions were typically small, encapsulated nodules of biliary ducts, mostly normal in structure and staining characteristics (Fig. 2.8).

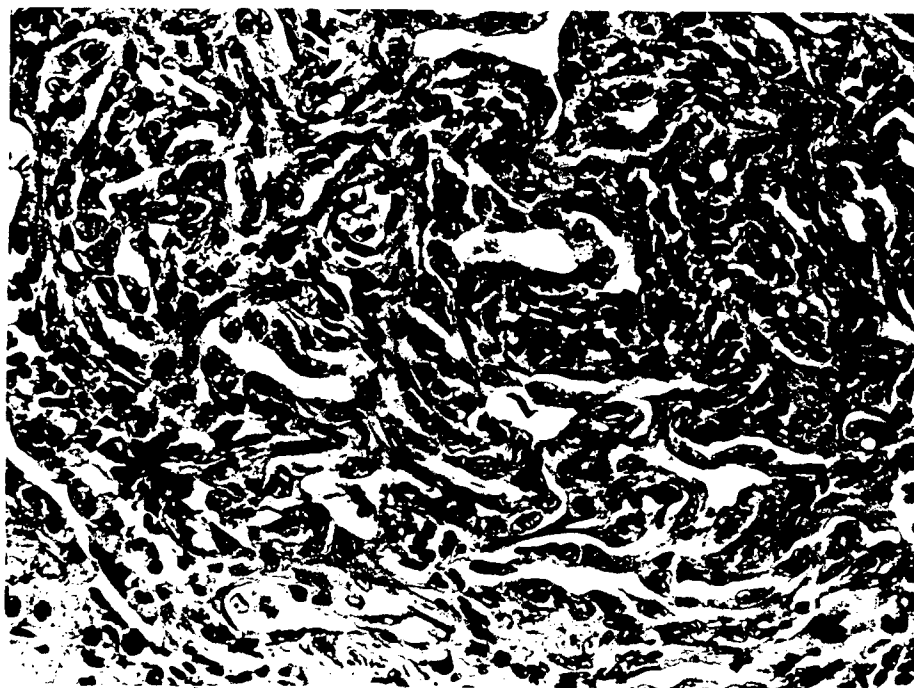


Fig. 2.8. A small cholangiocellular adenoma in a zebrafish exposed to 2500 ppm DMN as a fry. The lesion is well-encapsulated by connective tissue. H&E, X544.

Cholangiocellular carcinomas were the most numerous neoplasms observed with both nitrosamines. They were larger lesions with multilocular ducts, more frequent mitotic figures, and a lack of encapsulation (Fig. 2.9). An interesting variant

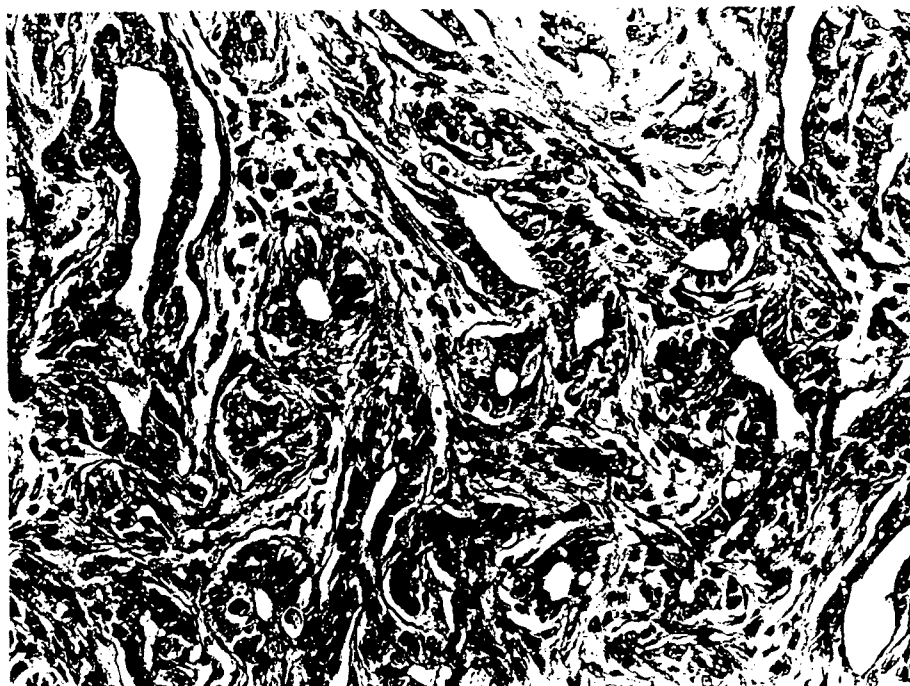


Fig. 2.9. A portion of a large cholangiocellular carcinoma in a zebrafish exposed to 1500 ppm DEN as a fry. H&E, X340.

of this tumor type had extensive replacement of the biliary ductal cells by rodlet cells (Fig. 2.10). The unanswered question of whether these cells are host or parasitic cells reemerges, but their appearance in these ducts does suggest differentiation into this cell type.

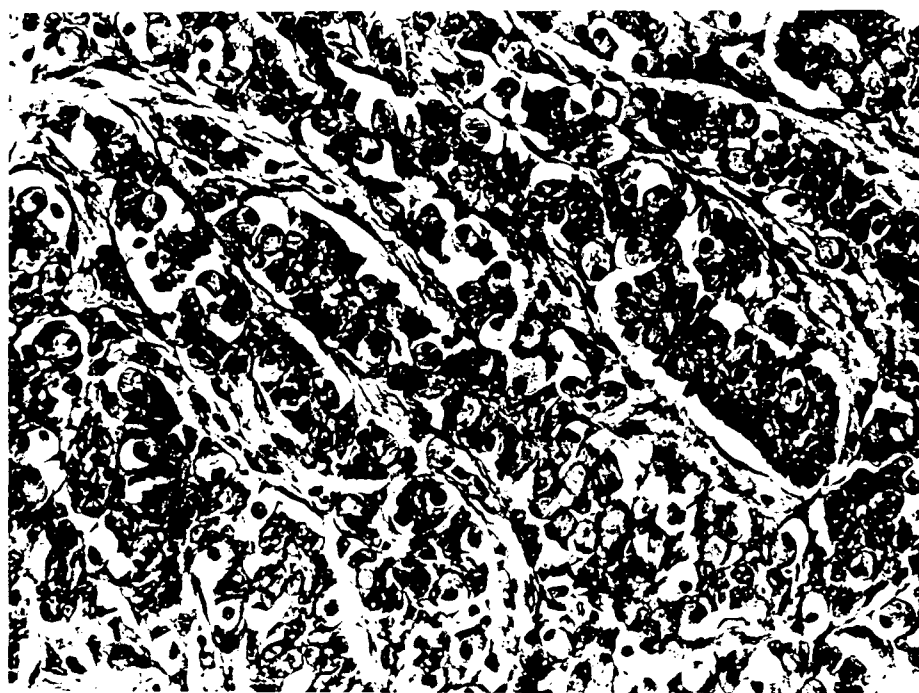


Fig. 2.10. Another area of the same carcinoma as in Fig. 2.9. In this area, rodlet cells (arrows) have replaced many of the biliary cells. In most cases the cells are oriented with their nuclei pointed toward the base or outside of the tubule. H&E, X544.

One non-epithelial neoplasm was observed in the liver of a 2500 ppm DMN-exposed fish, and was diagnosed as a hemangioma. The lesion was small, consisting of several capillary structures and surrounding connective tissue (Fig. 2.11).

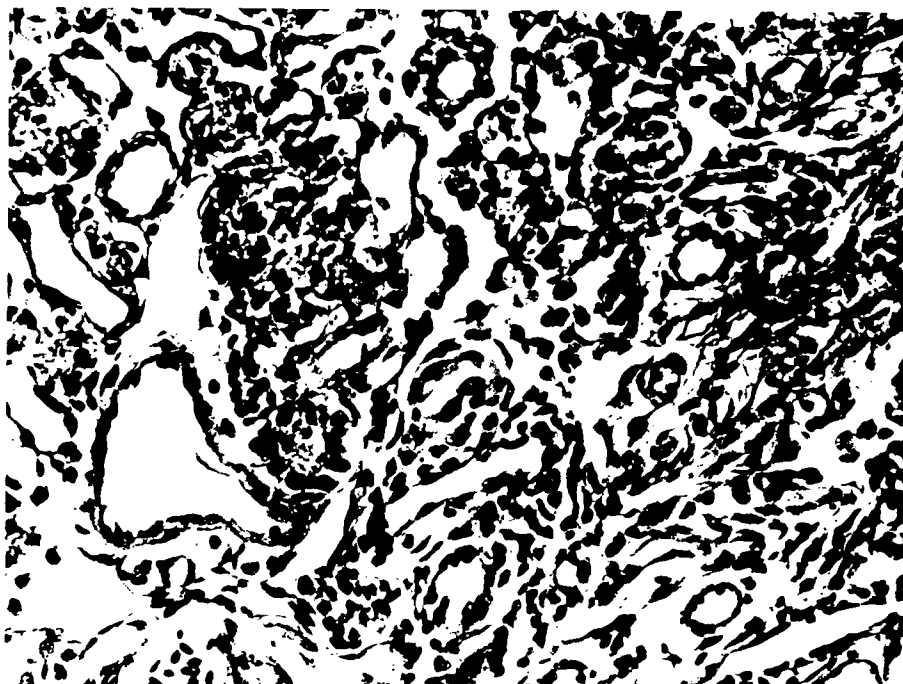


Fig. 2.11. A lesion of proliferating capillaries, diagnosed as a hemangioma, in the liver of a zebrafish exposed as a fry to 2500 ppm DMN. H&E, X544.

Several leiomyosarcomas were also found in the intestinal wall of the fish exposed to the two highest doses of DMN. They consisted of spindle-shaped cells forming solid or whorled patterns that invaded surrounding normal tissue (Fig. 2.12).



Fig. 2.12. A portion of a leiomyosarcoma in the wall of the intestine. Zebrafish exposed to 2500 ppm DMN as a fry. H&E, X340.

Embryo Bath Exposure

Zebrafish embryos were exposed only to DEN. The neoplastic response and mortality data are presented in Table 2.7. Treatment related mortalities and tumor incidences were lower, at comparable or higher doses, than for fry exposure to DEN. Mortalities differed significantly between treatments ($p < 0.0001$, 3 df). Over the three DEN doses (1000 to 3000 ppm) there was a significant linear dose response ($p < 0.001$, 1 df logistic regression) with no evidence of lack of fit to linear ($p > 0.5$, 1 df). Among those that survived, there were highly significant differences in neoplastic response between treatments ($p = 0.0031$, 3 df). The treatment differences can be explained by a highly significant difference between the controls and all of the lots getting at least some DEN dose ($p = 0.0005$, 1 df). Among lots receiving DEN, there was no evidence of differences due to dose ($p = 0.3937$, 2df). As with fry exposure to DEN, the liver was the primary target organ, and the same spectrum of hepatic tumors was seen with embryo as with fry exposure (Table 2.8).

Table 2.7. Carcinogenic response of zebrafish, exposed to static DEN solutions as 60 hr embryos, for 24 hr, and terminated 12 mo later

Lot	DEN dose ppm	Mortality % ^c	Neoplastic response	
			Inc. ^a	% ^b
1	Control	51	1/73	1
2	Control	55	1/68	1
1	1000	50	5/75	7
2	1000	53	5/70	7
1	2000	67	6/50	12
2	2000	65	6/52	11
1	3000	75	3/37	8
2	3000	80	4/30	13

^aNo. of fish with at least one neoplasm/total No. of fish

^bAmong the lots receiving DEN, there was no evidence of differences due to dose (p=0.3937, 2df)

^cMortalities differed significantly between treatments (p<0.0001, 3df)

Table 2.8. Neoplasia and associated lesions in zebrafish taken 1 yr after embryonic exposure to DEN^a

Lesion types	Lot	Total No. of tumors observed at each dose of DEN (ppm)			
		0	1000	2000	3000
CT ^b	1	-	-	-	-
	2	-	-	-	-
BH	1	-	-	5/50	-
	2	-	-	-	-
Liver lesions - altered foci					
BF	1	-	-	-	-
	2	1/68	-	-	-
EF	1	-	4/75	-	5/37
	2	-	4/70	4/52	1/30
CF	1	-	-	-	-
	2	1/68	-	-	2/30
Liver neoplasms					
HCA	1	1/73	4/75	3/50	1/37
	2	1/68	3/70	5/52	2/30
HCC	1	-	-	1/50	-
	2	-	-	-	-
CCA	1	-	-	-	1/37
	2	-	-	-	-

Table 2.8 (continued)

CCC	1	-	-	-	1/37
	2	-	-	1/52	-
Other types of neoplasia					
AC (intestine)	1	-	1/75	-	-
	2	-	-	-	-
CH (Spine)	1	-	-	-	-
	2	-	1/70	1/52	-
FS (operculum)	1	-	-	-	-
	2	-	1/70	-	-
CS (gill)	1	-	-	1/50	-
	2	-	-	-	-
UA	1	-	-	-	-
	2	-	-	-	1/30
LS (intestine)	1	-	-	-	-
	2	-	-	-	1/30
IP (nose)	1	-	-	-	-
	2	-	-	-	1/30

^aEmbryos given 24 hr immersion exposure to treatments at 60 hr post fertilization

^bAbbreviations used: CT - cytotoxicity, BH - biliary hyperplasia, BF - basophilic foci, EF - eosinophilic foci, CF - clear cell foci, HCA - hepatocellular adenoma, HCC - hepatocellular carcinoma, CCA - cholangiocellular adenoma, CCC - cholangiocellular carcinoma, AC - adenocarcinoma (intestine), CH - chordoma (spine), FS - fibrosarcoma (operculum), CS - chondrosarcoma (gill), UA - ultimobranchial adenoma, LS - leiomyosarcoma (intestine), IP - inverted papilloma (nose)

More examples of non-hepatic neoplasms occurred in the embryo-exposed groups. These included an intestinal adenocarcinoma in a fish from the 1000 ppm group (Fig. 2.13), two chordomas of the spine, one each in the 1000 and 2000 ppm groups (Fig. 2.14), a fibrosarcoma of the operculum in the 1000 ppm group (Figs. 2.15 & 2.16), a chondrosarcoma located in the gill in the 2000 ppm group (Fig. 2.17), an ultimobranchial adenoma in the 3000 ppm group (Figs. 2.18 & 2.19), a leiomyosarcoma of the intestine in the 3000 ppm group (Fig. 2.20), and an inverted papilloma on the skin of the nose in the 3000 ppm group (Fig. 2.21).

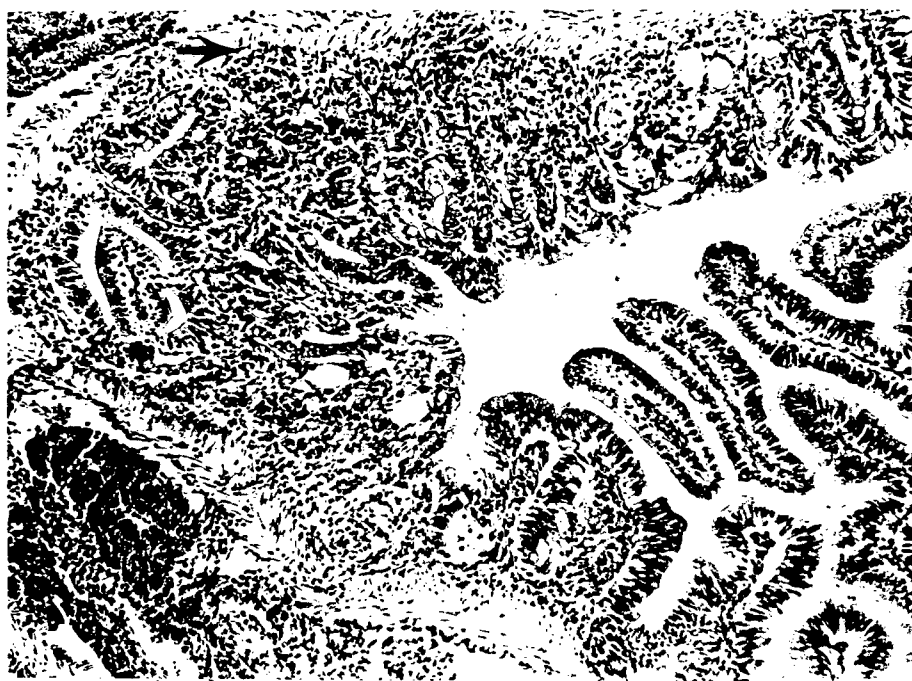


Fig. 2.13. A small adenocarcinoma of the intestine. Invasion of the tunica muscularis is evident focally (arrow). Embryonic exposure to 1000 ppm DEN. H&E, X136.



Fig. 2.14. A chordoma invading the spinal cord. Embryonic exposure to 1000 ppm DEN. H&E, X136.



Fig. 2.15. A fibrosarcoma of the operculum. Locally there is extensive necrosis deep in the neoplasm (arrows). Embryonic exposure of zebrafish to 1000 ppm DEN. H&E, X54.

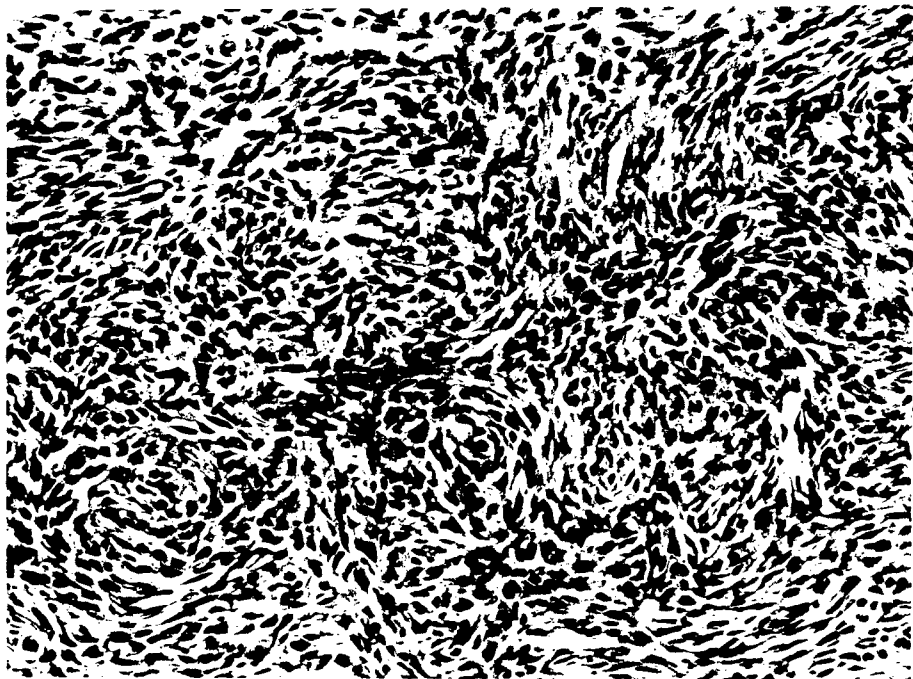


Fig. 2.16. Greater detail of the fibrosarcoma of Fig. 2.16. Anaplastic spindloid to stellate fibroblasts are randomly arranged and set in a scant fibrovascular stroma. H&E, X340.



Fig. 2.17. Chondrosarcoma of the gill in a zebrafish exposed to 2000 ppm DEN as an embryo. Clusters of embryonal blast cells as well as islands of more differentiated chondrocytes are present. H&E, X340.

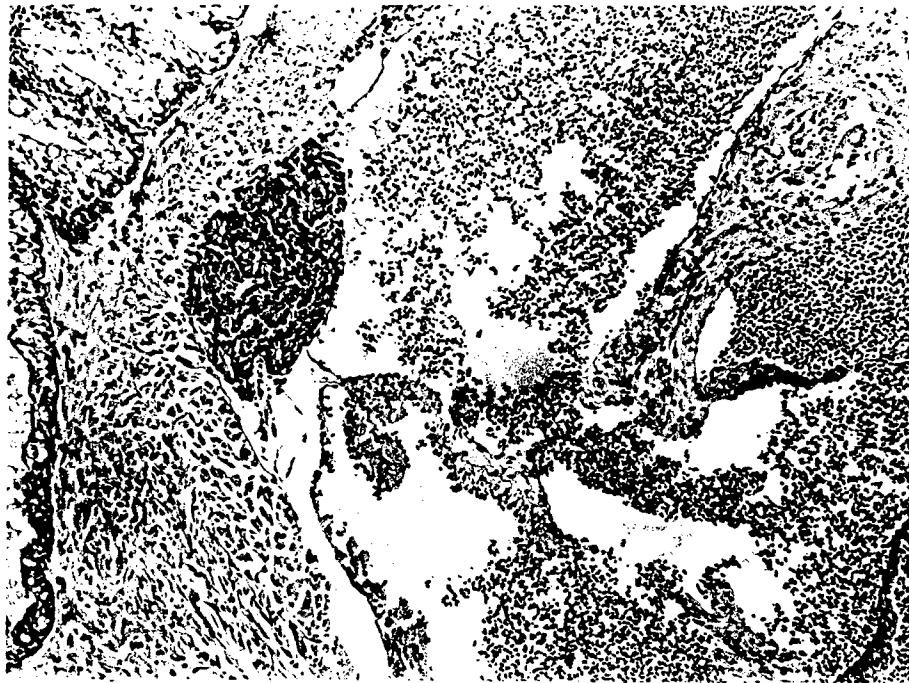


Fig. 2.18. Normal ultimobranchial gland in the transverse septum between the heart and the esophagus. Follicles are lined by columnar epithelial cells with abundant eosinophilic cytoplasm and basal nuclei. Control zebrafish. H&E, X136.



Fig. 2.19. Adenoma of the ultimobranchial gland with cross-sectional area over 5X normal size. Loss of distinct acinar arrangement of epithelial cells is evident, with ovoid to polygonal epithelial cells containing scant eosinophilic cytoplasm forming irregular cords, monotonous sheets and occasional acini. Embryonic exposure of zebrafish to 3000 ppm DEN. H&E, X136. Inset X544.

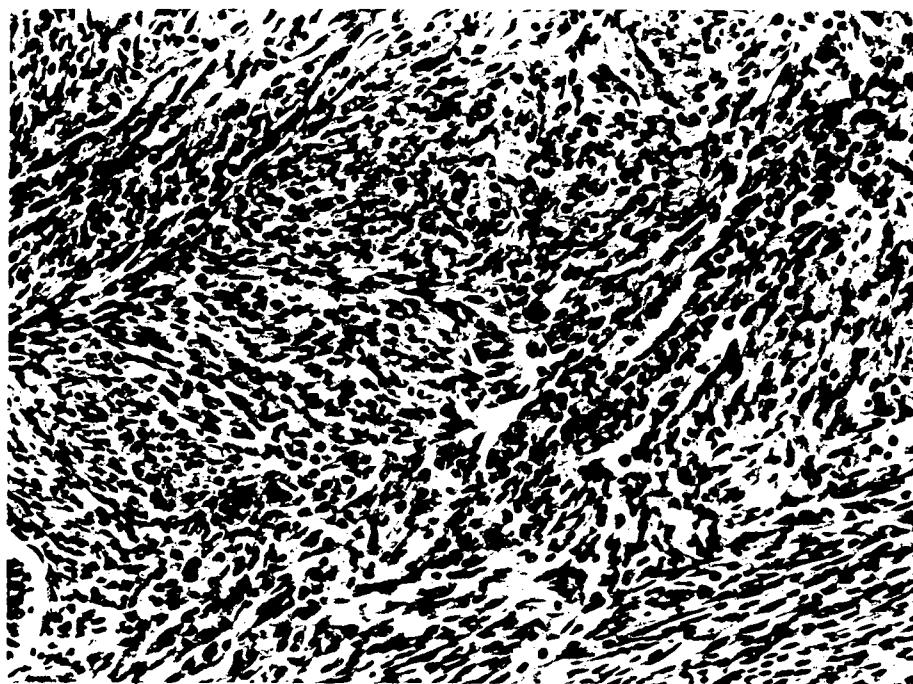


Fig. 2.20. Leiomyosarcoma forming a transmural mass in the intestine and invading adjacent peritoneum. Interlacing bundles of spindloid myocytes, often with cigar-shaped nuclei, and scant to abundant fibrillar eosinophilic cytoplasm comprise the bulk of the mass, with areas of less differentiated ovoid to stellate cells containing pleomorphic nuclei also present. Embryonic exposure of zebrafish to 3000 ppm DEN. H&E, X340.



Fig. 2.21. An inverted squamous papilloma on the anterior aspect of the upper jaw. Multilobulated mass in dermis is comprised of well-differentiated epidermis containing mucous cells. Zebrafish exposed to 3000 ppm DEN as an embryo. H&E, X340.

DISCUSSION

Dietary exposure of aquarium fish to carcinogens has been used sparingly in comparison to water exposures (Khudoley, 1972; Sato *et al.*, 1973; Pliss and Khudoley, 1975; Hatanaka *et al.*, 1982). Most of these experiments have used water insoluble compounds such as aflatoxins, heterocyclic amines, azo dyes, or polycyclic aromatic hydrocarbons, and have produced significant numbers of hepatic neoplasms in both guppies and medakas. Sato *et al.* (1973), however, did feed DMN to guppies at 4,800 ppm for 13 mo, and saw only a minimal response, 2 of 20 fish had what were described as hyperplastic nodules in the liver. The total lack of response to DMN and DEN after 12 wk of exposure to high doses of these compounds may be the result of several factors: 1) insufficient exposure time and/or dose. The only dietary exposures of fish to these carcinogens, for comparison, have been the study by Sato *et al.* (1973), where both a higher dose and longer exposure to DMN gave a minimal response, and with rainbow trout, in which lower doses but longer exposure times of either 9 or 12 mo gave high incidences of liver tumors (Grieco *et al.* 1978; Hendricks *et al.*, 1994). The shorter exposure time used in the current experiment was based on the higher doses used compared to the trout studies, the shorter life span of zebrafish compared to trout which could translate into a shorter response time, and facility demands which prevented longer term exposures, 2) age of the fish when exposure began. The guppies used by Sato *et al.* (1973) were less than one mo old. In rainbow trout, we have observed decreasing sensitivity to carcinogen exposure with increasing age (unpublished observations), thus the fact that our

zebrafish were too old and near sexual maturity, may have reduced their sensitivity, and 3) possible pharmacokinetic differences in zebrafish that limit absorption and distribution of dietary-delivered carcinogens, specifically nitrosamines. The lack of a stomach in zebrafish may alter digestion, absorption, and delivery of carcinogens to the liver as compared with the rainbow trout, a fish that responds with high sensitivity to dietary carcinogen exposures.

The spectrum of hepatic neoplasms produced by DEN and DMN in the water-borne exposures of both fry and embryos, were similar to those reported by authors exposing several other species of small fish to DEN (Stanton, 1965; Schultz and Schultz, 1982a; Parland and Baumann, 1985; Couch and Courtney, 1987; and Bunton, 1989). One lesion that has been reported in medaka and sheepshead minnows, in response to water-borne DEN, is spongiosis hepatitis (Hinton *et al.*, 1984; Couch and Courtney, 1987). This lesion has not been seen thus far in any of our zebrafish samples.

Perhaps the most interesting result from these exposures of zebrafish to nitrosamines is the unusual or rare tumor types observed in the fish exposed to DEN as embryos. Although few in number, the chordomas of the spine, the chondrosarcoma of the gill and the ultimobranchial adenoma are tumor types that have not been previously described from DEN exposures to aquarium fish. The only other known embryo exposure of aquarium fish to a carcinogen was reported by Klaunig *et al.* (1984). They exposed medaka to DEN at concentrations of 25, 50, and 100 ppm for 10 days, and found liver tumors in 4, 15, and 43% of the fish, respectively, after six months. It is not possible to compare the relative sensitivities of medaka and

zebrafish in these two studies. However, it appears that medaka were more responsive to hepatocarcinogenesis, but no other tumor types were reported. They used lower doses for a longer time, something we could not do with zebrafish, since the entire developmental period from fertilization to hatch is only 96 hr.

The overall low response of zebrafish to the hepatocarcinogenicity of strong carcinogens such as DEN and DMN, may be due to inherent genetic factors in zebrafish and other members of the Cyprinidae family. Other members of that family have demonstrated dramatic resistance to carcinogenesis in both controlled laboratory experiments (*Pimephales promelas*, the fathead minnow) and highly polluted natural environments (*Cyprinus carpio*, the common carp) (Hawkins *et al.*, 1988a). It is possible that fish of this family have specific digestive, pharmacokinetic, metabolic, DNA repair, tumor suppressor gene, or other mechanisms that provide protection against carcinogenesis. Future experiments will attempt to explore some of these unknowns.

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Chapter 3

THE CARCINOGENICITY OF DIETARY AFLATOXIN B₁ TO ZEBRAFISH (*BRACHYDANIO RERIO*) AND THE POSTINITIATION EFFECTS OF DEHYDROEPIANDROSTERONE

Hsi-Wen Tsai¹ and Jerry D. Hendricks^{1,2}

¹Department of Food Science and Technology

²Marine/Freshwater Biomedical Sciences Center

Oregon State University, Corvallis, OR

ABSTRACT

Duplicate groups of zebrafish (*Brachydanio rerio*) were exposed to dietary aflatoxin B₁ (AFB₁) at doses of 10, 20, or 30 ppm for 3 mo and then fed either the control diet or the control diet plus 444 ppm dehydroepiandrosterone (DHEA) for an additional 6 mo. Histological examination of tissues revealed a low but dose responsive incidence of hepatic neoplasms in the AFB₁-exposed fish, and a slight but non-significant promoting effect by DHEA. The responses are compared to results observed in rainbow trout and discussed with respect to AFB₁ metabolism in the two species.

has been shown to be a powerful inhibitor of AFB₁ carcinogenesis in rainbow trout when administered prior to or during AFB₁ exposure (Dashwood *et al.*, 1988; Dashwood *et al.*, 1989), but an equally potent promoter of AFB₁ carcinogenesis when fed after AFB₁ exposure (Bailey *et al.*, 1987; Dashwood *et al.*, 1991). Chlorophyllin, extracted from spinach, also was effective as a pre-initiation inhibitor, but showed no promoting properties after AFB₁ initiation in trout (Breinholt *et al.*, 1995). Dehydroepiandrosterone (DHEA), a naturally-occurring steroid hormone in vertebrates, proved to be not only a promoter of AFB₁ carcinogenesis in rainbow trout but also a complete carcinogen on its own (Orner *et al.*, 1995).

An intermediate between pregnenolone and testosterone in the steroid biosynthetic pathway, DHEA is produced primarily in the adrenal cortex and is the steroid found in the highest concentration in human plasma (Schwartz *et al.*, 1988). Beyond its function as an intermediate in steroid biosynthesis, its physiological role is unclear (Gordon *et al.*, 1987). It has been reported to have a number of beneficial anti-aging, anti-obesity, anti-diabetic, anti-atherogenic, and anti-carcinogenic effects (Cleary, 1990; Cleary *et al.*, 1984; Cleary *et al.*, 1983; Gordon *et al.*, 1987; Moore *et al.*, 1986; Nyce *et al.*, 1984; Pashko *et al.*, 1985; Schwartz, 1979; Schwartz and Tannen, 1981; Weber *et al.*, 1988) in mammalian experimental animals. Conversely, DHEA has been shown to give rise to hepatic neoplasms in rats (Rao *et al.*, 1992a; Rao *et al.*, 1992b; Metzger *et al.*, 1995) and in rainbow trout as cited above.

Our current goals are to determine the responsiveness of zebrafish to various classes of carcinogens, in this case aflatoxins, by multiple exposure routes, and also to explore the modulation of carcinogenesis by compounds which have environmental,

dietary, or physiological significance. Based on the results of DHEA in rainbow trout, we decided to use this compound for our first experiments on modulation of dietary AFB₁ carcinogenesis in zebrafish.

MATERIALS AND METHODS

Chemicals

DHEA and AFB₁ were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental animals

Zebrafish (*Brachydanio rerio*) were spawned and raised at the Food Toxicology and Nutrition Laboratory, Oregon State University as previously described (Tsai *et al.*, 199X). Animals were maintained in 110 L glass tanks supplied with processed well water (Tsai *et al.*, 199X) and a 14-hr light and 10-hr dark photoperiod.

Carcinogen exposure

Two mo after spawning, duplicate groups of 90 fish were started on Modified Purified Casein diet (MPC) (Tsai *et al.*, 199X), or MPC containing 10, 20, or 30 ppm AFB₁ and fed for three mo. At that time, one of the duplicate groups from each treatment was started on MPC containing 444 ppm DHEA and fed this diet for an additional six mo. The other group from each treatment received MPC only. At the end of the six-mo promotional phase of the experiment, all the fish were killed, fixed in Bouin's solution, and processed for histology as described in Tsai *et al.* (199X). Tumor detection was based on three histological sections per fish only. Thus, some small neoplasms could have been missed.

Statistics

Tumor incidence data were analyzed by logistic regression with categorical and/or continuous predictors in the Genmod procedure of SAS (SAS, 1996). Further, for this experiment, which had no replication, there was no evidence of lack of fit after a linear dose response was fit, although the degrees of freedom were small.

RESULTS

Mortality was low in the control groups but increased with AFB₁ dose (Table 3.1). Among those that survived, there was little evidence of a DHEA effect on tumor incidence ($p=0.1520$, 1 df), but there was a significant effect due to AFB₁ dose ($p=0.0004$, 2 df). Over the three AFB₁ doses (10 to 30 ppm) there was a significant linear dose response ($p<0.0001$, logistic regression, 1 df) with no evidence of lack of fit to linear ($p>0.5$, 1 df). DHEA was not carcinogenic at the 444 ppm level when fed to control zebrafish.

Table 3.1. Effect of DHEA on mortality and tumor incidence when fed after dietary AFB₁ exposure^a

Dietary additions ^b		Lot	Mortality %	Neoplastic Response	
AFB ₁ ppm	DHEA ppm			Inc. ^{c,d}	%
0	0	1	1	0/89	0
0	444	2	1	0/89	0
10	0	3	12	1/78	1
10	444	4	23	2/67	3
20	0	5	28	3/62	5
20	444	6	35	3/55	6
30	0	7	30	6/60	10
30	444	8	32	11/58	19

*Abbreviations used: AFB₁ - aflatoxin B₁; DHEA - dehydroepiandrosterone

^bAFB₁ and DHEA were added to the modified purified casein diet, AFB₁ was fed for mo 1-3, DHEA for mo 4-9

^cNo. of fish with tumors/total No. of surviving fish

^dThere is little evidence of a DHEA effect on tumor incidence ($p=0.1520$, 1 df), but there is a significant effect due to AFB₁ dose ($p=0.0004$, 2 df)

Gill epithelial hyperplasia was the only consistent non-hepatotoxic response observed in the treatment groups, but was not dose-responsive (Figs. 3.1 & 3.2). There was no evidence of infectious agents or inflammatory reactions in any of the fish. Biliary hyperplasia (Fig. 3.3) was seen sporadically throughout the treated groups, apparently without a dose dependence.



Fig. 3.1. Normal gill filaments from a control zebrafish. The epithelium covering the lamellae is a single squamous layer. H&E, X136.



Fig. 3.2. Hyperplastic gill epithelium from a zebrafish fed the 20 ppm dose of AFB₁. Hyperplastic cells are cuboidal in shape and most numerous at the bases of the lamallae. H&E, X340.

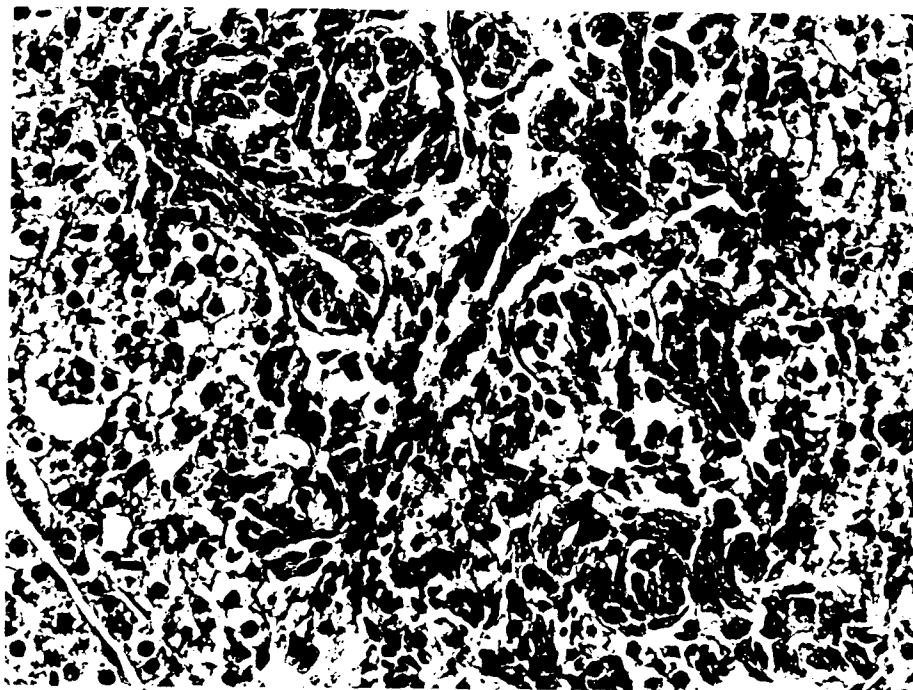


Fig. 3.3. A focal area of bile duct hyperplasia along a biliary tract from a zebrafish fed the 30 ppm dose of AFB₁. H&E, X544.

The foci of altered hepatocytes and neoplastic hepatic lesions that resulted from AFB₁ or combined AFB₁/DHEA exposure were similar to those previously described in zebrafish exposed to nitrosamines (Tsai *et al.*, 199X) (Table 3.2).

Table 3.2. Numbers of neoplastic and associated lesions in zebrafish taken after 3 mo of dietary AFB₁ exposure and 6 mo of dietary DHEA exposure^a

Dietary additions ^b		Total No. of lesions in each lot						
AFB ₁ ppm	DHEA ppm	BF	EF	CF	CCA	CCC	HCA	HCC
0	0	-	-	-	-	-	-	-
0	444	-	-	-	-	-	-	-
10	0	-	1/78	-	-	1/78	-	-
10	444	-	2/67	-	1/67	1/67	-	-
20	0	1/62	3/62	1/62	-	1/62	-	1/62
20	444	2/55	1/55	-	-	2/55	-	1/55
30	0	1/60	1/60	-	1/60	4/60	1/60	1/60
30	444	4/58	2/58	1/58	1/58	5/58	-	5/58

^aAbbreviations used: AFB₁ - aflatoxin B₁; DHEA - dehydroepiandrosterone; BF - basophilic foci; EF - eosinophilic foci; CF - clear cell foci; CCA - cholangiocellular adenoma; CCC - cholangiocellular carcinoma; HCA - hepatocellular adenoma; HCC - hepatocellular carcinoma

^bAFB₁ and DHEA were added to the modified purified casein diet, AFB₁ was fed for mo 1-3, DHEA for mo 4-9

Basophilic, eosinophilic (Fig. 3.4) and clear cell (Fig. 3.5) foci were observed in a few of the fish, some of which also had well-developed neoplasms. Basophilic foci were small with cells that were normal in appearance with the exception of reduced glycogen and cytoplasmic basophilia. Eosinophilic foci were the most numerous of the foci and occurred at all dose levels. The cells were enlarged with decreased glycogen and more intense eosinophilia (Fig. 3.4). Clear cells were enlarged and had extensive vacuolation. Nuclei were usually centric in location (Fig. 3.5).

Benign neoplasms included cholangiocellular adenomas and hepatocellular adenomas. The cholangiocellular adenomas were small lesions of encapsulated, mostly normal appearing bile ducts (Fig. 3.6). Hepatocellular adenomas were small, either eosinophilic or basophilic, with normal tubular structure, but with increased cellular size and reduced glycogen levels (Fig. 3.7).

Malignant neoplasms were cholangiocellular carcinomas, which were large lesions with numerous, multilocular ducts, associated connective tissue, and were invasive to surrounding tissue (Fig. 3.8), and hepatocellular carcinomas. The latter tumors were large, basophilic, and composed of mostly uniform, well-differentiated cells that proliferate within the original hepatic tubules and transform them into a multicellular sacs of neoplastic cells between adjacent sinusoids (Fig. 3.9). One fish from the 20 ppm AFB₁ group had a neoplasm located in the pancreas. It consisted primarily of ductal structures, and was diagnosed as an adenocarcinoma of pancreatic ductal epithelium, with no acinar involvement (Fig. 3.10).

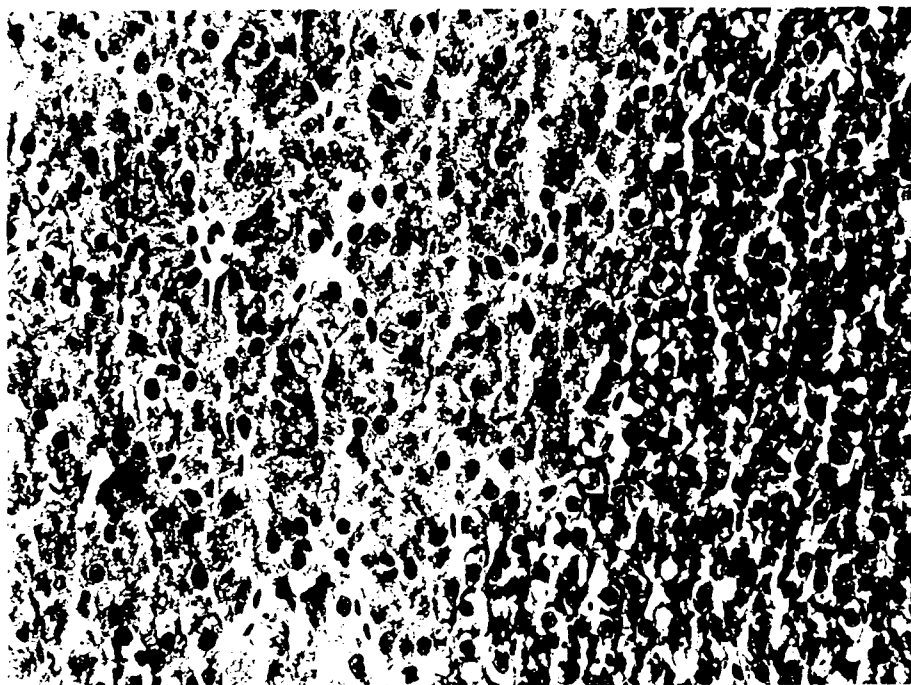


Fig. 3.4. An eosinophilic focus in the liver of a zebrafish fed 20 ppm AFB₁ and 444 ppm DHEA. The hepatocytes are enlarged, with irregular nuclei, reduced glycogen content, and granular, eosinophilic cytoplasm. H&E, X544.

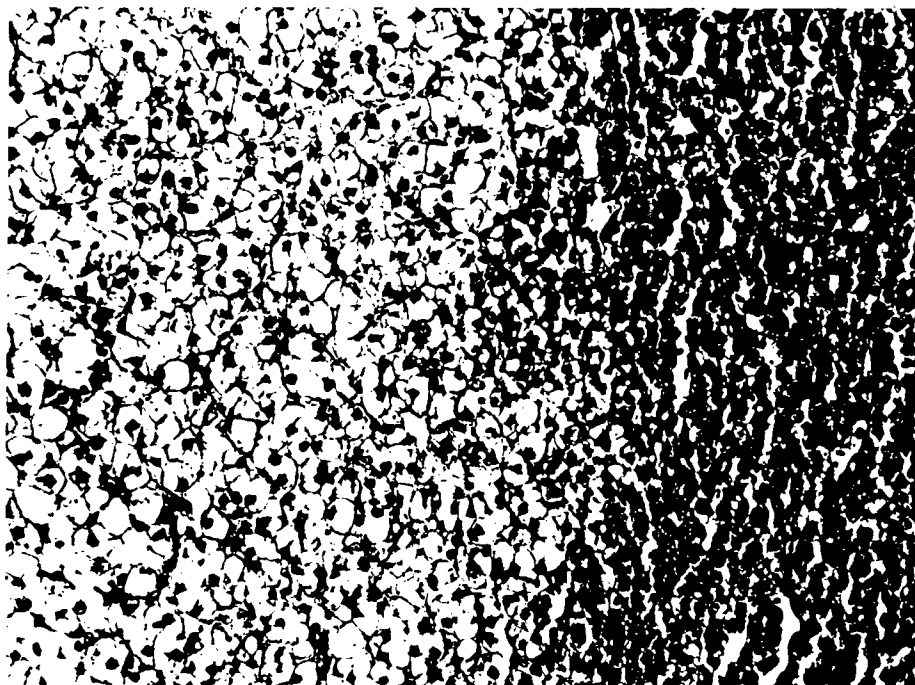


Fig. 3.5. A clear cell focus in a zebrafish fed 30 ppm AFB₁ & 444 ppm DHEA. Hepatocytes are enlarged and extensively vacuolated. H&E, X340.



Fig. 3.6. A small cholangiocellular adenoma in the liver of a zebrafish fed 30 ppm AFB₁. The biliary ducts are normal in appearance and surrounded by connective tissue. H&E, X340.

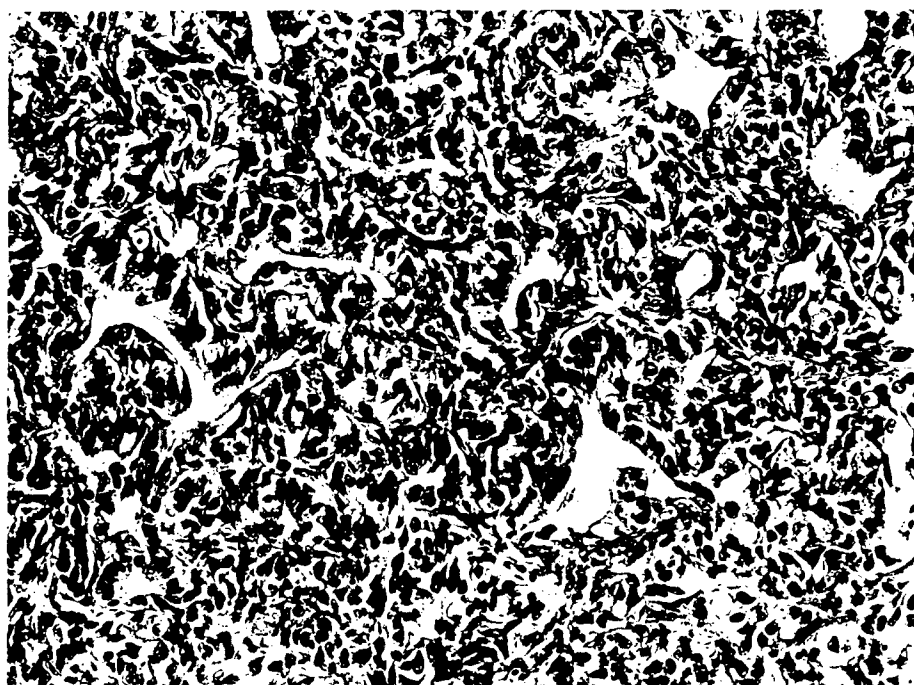


Fig. 3.7. A portion of a large cholangiocellular carcinoma from a zebrafish fed 20 ppm AFB₁ and 444 ppm DHEA. The ducts are irregular and branching, non-encapsulated and invasive to surrounding liver (bottom). H&E, X340.

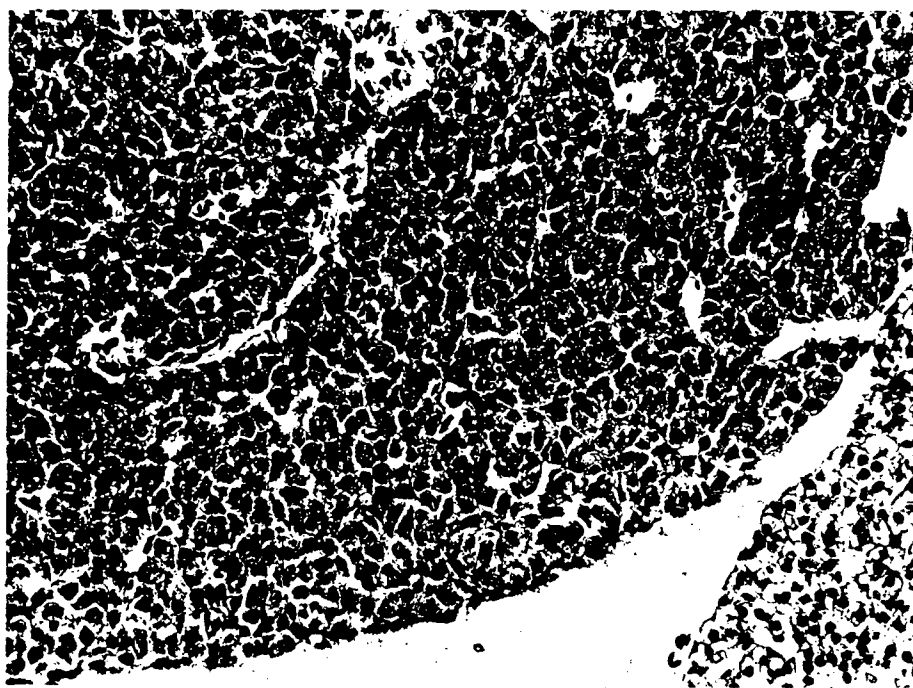


Fig. 3.8. A basophilic hepatocellular adenoma from a fish fed 30 ppm AFB₁. Cells are slightly enlarged, low in glycogen and still occur in a normal tubular arrangement. H&E, X340.

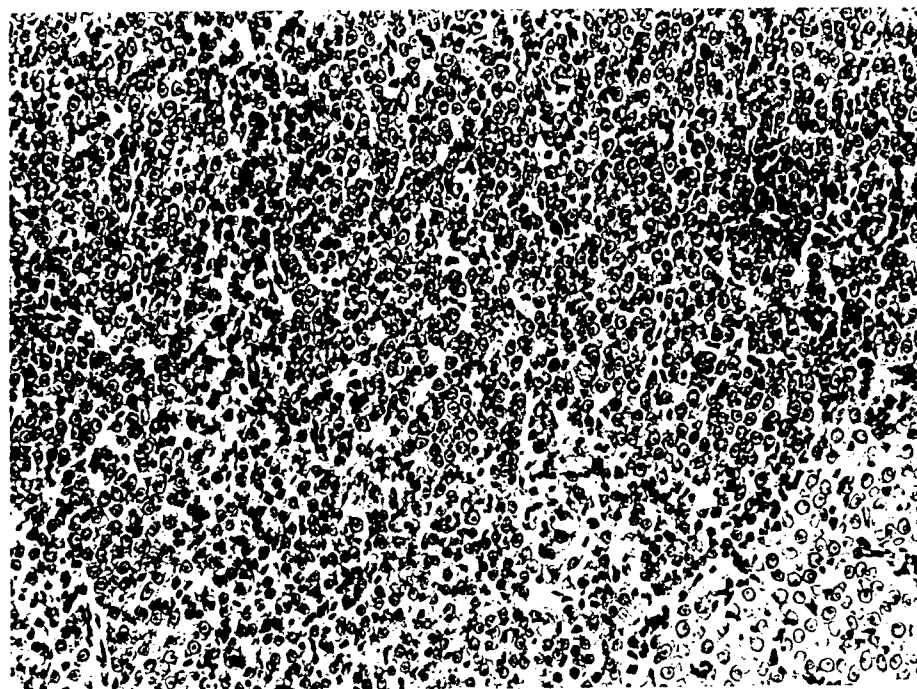


Fig. 3.9. A portion of a large hepatocellular carcinoma in a zebrafish fed 20 ppm AFB₁. Neoplastic cells are small, uniform and have disrupted the normal tubular architecture. H&E, X340.

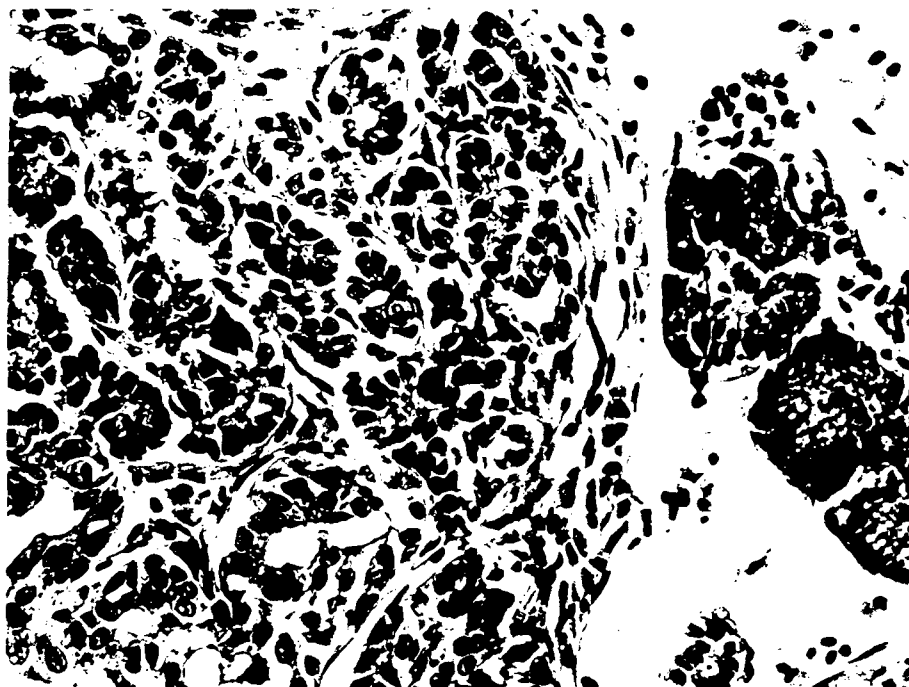


Fig. 3.10. A ductal adenocarcinoma in the pancreas of a zebrafish fed 20 ppm AFB₁. Normal pancreatic acinar cells are at the right. H&E, X544.

DISCUSSION

These results confirm previous reports in the literature and preliminary experimental data generated in our laboratory, that zebrafish are surprisingly resistant to the carcinogenicity of AFB₁. The work of Troxel *et al.* (199X) has investigated the metabolism and DNA-binding of AFB₁ in zebrafish and provides some hypotheses for this resistance. Troxel *et al.* (199X) found that zebrafish begin to rapidly excrete the metabolite, aflatoxicol (AFL), within 5 min of an intraperitoneal injection of AFB₁, and within 24 hr, 47% of the administered radioactivity had been excreted into the water. After 18 hr, AFL-glucuronide became the major metabolite excreted. Thus, zebrafish have an extremely fast-acting cytosolic reductase system that begins generating AFL within 5 min and have the ability to excrete this metabolite directly into the water, presumably through the gills. Later, phase II metabolism takes over and AFL-glucuronide is excreted, probably through the urine. Both of these mechanisms could function to reduce the available AFB₁ that could be metabolized to the epoxide for binding to DNA, and provide protection against AFB₁ carcinogenesis.

Supportive of this hypothesis is the 4-fold lower DNA binding of AFB₁ in zebrafish in comparison with rainbow trout (Troxel *et al.*, 199X). They also found that female zebrafish bind approximately twice as much AFB₁ to liver DNA as males. We were interested to see if this increased DNA binding in females would result in a higher incidence of neoplasms in females than males. However, when we noted the sex of the tumor-bearing fish, there was no evidence of a sex effect. Of the 26 total tumor-bearing fish, 14 were males and 12 were females. At the high dose of AFB₁,

three males and three females had tumors, and when DHEA was added, six males and five females bore tumors. Buchmann *et al.* (1993) showed that the cytochrome P450 isozyme (CYP2K1), thought to be responsible for bioactivation of AFB₁, is constitutively expressed in zebrafish, providing a basis for the response that we do observe.

Another variable that effects the sensitivity of rainbow trout to carcinogens is age. We have found that young rainbow trout (embryos, and small fry) are much more susceptible to AFB₁ carcinogenesis than are older fish (Hendricks, unpublished observations). The zebrafish used in this feeding trial were adults or subadults (2-month old) when the dietary exposure began, and thus they could possibly have been more responsive if they had been younger. However, the results of Troxel *et al.* (199X) were also obtained on adult fish, so their results do directly apply to ours.

Although there appeared to be some slight effect of DHEA as a promoter, its effects were much less than in rainbow trout, and DHEA demonstrated no initiating activity at a dose that was clearly carcinogenic to rainbow trout (Orner *et al.*, 1995). In rodents, DHEA is a peroxisomal proliferator, and it is thought to exert its carcinogenic properties through poorly understood mechanisms involving peroxisome proliferation (Rao *et al.*, 1992b). In rainbow trout, however, which respond poorly to peroxisome proliferators, carcinogenesis and promotion occurred in the absence of this mechanism (Orner *et al.*, 1995). Orner *et al.* (1995) suggested that the promotional mechanism of DHEA may be due to its conversion into excess estrogens, which are promoters of carcinogenesis in rainbow trout (Nunez *et al.*, 1989). We do

not know how zebrafish respond to peroxisome proliferators, but would assume that their response would be more like trout than rodents.

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Chapter 4

THE CARCINOGENICITY OF METHYL AZOXYMETHANOL ACETATE TO ZEBRAFISH (*BRACHYDANIO RERIO*) WITH A COMPARISON TO JAPANESE MEDAKA (*ORYZIAS LATIPES*)

Hsi-Wen Tsai¹, Jan Spitsbergen¹,
Daniel Arbogast¹, and Jerry Hendricks^{1,2}

¹Department of Food Science and Technology

²Marine/Freshwater Biomedical Sciences Center

Oregon State University, Corvallis, OR

ABSTRACT

Zebrafish (*Brachydanio rerio*) were exposed to methylazoxymethanol acetate (MAM-Ac) by three different exposure routes: dietary, fry and embryo static water bath. In addition, a comparison between zebrafish and Japanese medaka (*Oryzias latipes*), with respect to carcinogenic sensitivity to dietary MAM-Ac, was made.

With all three exposure routes, the liver was the primary target organ for neoplasms, and the usual array of altered foci, benign and malignant neoplasms of both hepatic and biliary cells was observed. In addition to hepatic tumors, single tumors at diverse organ sites occurred with all the routes of exposure, but the greatest variety came from the embryonic water bath exposure. In the comparative experiment between zebrafish and medaka, the overall tumor incidences were very similar, but medaka had tumors in the liver only whereas the zebrafish had fewer liver tumors but several extrahepatic neoplasms.

INTRODUCTION

Zebrafish (*Brachydanio rerio*) were the first small aquarium fish to be used for carcinogenesis studies. Stanton (1965) reported on the carcinogenicity of diethylnitrosamine in this species, and also exposed zebrafish to cycad nut meal in the diet and cycasin in the water (Stanton, 1966). Since these early experiments by Stanton, only Pliss and Khudoley (1975) and Khudoley (1984), also using nitrosamines, have reported on carcinogenicity experiments with zebrafish.

We have undertaken an investigation to determine if this species has utility as a model for carcinogenesis, and have already reported on experiments using multiple exposure routes for nitrosamines (Tsai *et al.*, 199X), and dietary exposure to aflatoxin B₁ (Tsai and Hendricks, 199X). Methylazoxymethanol acetate (MAM-Ac), a stable form of MAM, the active molecule in cycasin and the cycad nut, has been used extensively, as a model carcinogen, with Japanese medaka (*Oryzias latipes*) (Aoki and Matsudaira, 1977; 1981; Hatanaka *et al.*, 1982; Hawkins *et al.*, 1985; Hawkins *et al.*, 1986; Hawkins *et al.*, 1988; Harada *et al.*, 1988;), resulting in neoplasms in a number of different tissues and organs. In zebrafish, the one report on the effects of cycasin and cycad nut meal reported only liver tumors (Stanton, 1966). In this report, we present the results of dietary, fry and embryo water bath exposures of zebrafish to MAM-Ac, and compare the response of zebrafish and medaka to two doses of dietary MAM-Ac.

MATERIALS AND METHODS

Chemicals

Methylazoxymethanol acetate (MAM-Ac) was purchased from Sigma Chemical Company, St. Louis, MO.

Animals

Zebrafish used in this study were the progeny of stock fish obtained from 5-D Tropical Fish, Plant City, FL. Japanese medaka were obtained from Carolina Biological Supply Company, Burlington, NC. Fish culture procedures at our laboratory have been previously described (Tsai *et al.*, 199X). Animals were fed as described in Tsai *et al.*, 199X.

Dietary Exposure

MAM-Ac at doses of 500, 1000, or 2000 mg/kg (ppm) dry diet was dissolved in hot water (55°C), and together with the lipid fraction, was thoroughly mixed with the dry ingredients of the modified purified casein diet (MPC) (Tsai *et al.*, 199X). Duplicate groups of 90, 8-wk-old zebrafish were fed the different diets for 12 wk. They were then maintained on the MPC diet for another 12 wk when they were terminated to determine the neoplastic response. The sampling, tissue fixation, and slide preparation procedures have been previously described (Tsai *et al.*, 199X). Single groups of 100 medaka, 8-wk-old, were fed the MPC diet containing the 1000 and 2000 ppm doses of MAM-Ac for 12 wk and handled the same way as the zebrafish.

Controls were fed the MPC diet only and also handled the same way as the experimental fish.

Fry Bath Exposure

Single groups of 100 zebrafish fry, 21 days post-hatch, were exposed to MAM-Ac, dissolved directly in buffered water at concentrations of 6.25, 12.5, 25, 50, 75, or 100 ppm, under static conditions for 2 hr. Control fish were handled the same way without exposure to MAM-Ac. After exposure, the fish were placed in 110 L tanks, fed OTD and brine shrimp for 12 mo and terminated as previously described.

Embryo Bath Exposure

Duplicate groups of 150 zebrafish embryos, 72 hr postfertilization, were exposed to static, buffered water solutions of 0, 10, 25, and 50 ppm MAM-Ac for 12 hr. After exposure they were transferred to clean, buffered water in 1.5 L beakers, where they were allowed to hatch and begin feeding. Finally they were placed in 110 L tanks, fed OTD and brine shrimp for 12 mo, and terminated to determine neoplastic response. Slide preparation for histological analysis has been previously described.

Statistics

Tumor incidence data were analyzed by logistic regression with categorical and/or continuous predictors in the Genmod procedure of SAS (SAS, 1996). For experiments with replicate lots, there was no evidence of overdispersion ($p > 0.5$, all lack of fit tests) and residuals appeared consistent with the binomial error model.

Further, for the experiments which had no replication, there was no evidence of lack of fit after a linear or quadratic dose response was fit, although the degrees of freedom were small.

RESULTS

Dietary Exposure - zebrafish

Table 4.1 summarizes the results of the dietary exposure of zebrafish to MAM-Ac. The fish tolerated all the doses well. Feeding behavior and growth were similar in all groups. Mortalities were slightly elevated at the highest dose. Among those that survived, there were highly significant differences in tumor incidence between treatments ($p < 0.001$, 3 df). Among the three MAM-Ac doses (500 to 2000 ppm) the response increases with dose and the large increase between 1000 and 2000 ppm results in significant curvilinearity in the dose response ($p = 0.0047$, quadratic term).

Table 4.1. Carcinogenic response of zebrafish to dietary MAM-Ac^a.

Lot	MAM-Ac dose ppm	Mortality %	Neoplastic response Inc. ^c	% ^d
1	0	8	0/82	0
2	0	9	0/81	0
3	500	17 ^b	0/73	0
4	500	25 ^b	0/65	0
5	1000	7	5/83	6
6	1000	8	8/82	9
7	2000	11	23/79	29
8	2000	17	16/73	22

^aFish were fed MAM-Ac diets for 12 wk and terminated 24 wk after the start of the exposure

^bUnexplained loss of fish

^cNo. of tumor bearing fish/total No. of fish

^dThere were highly significant differences in tumor incidence between treatments ($p < 0.0001$, 3df)

Livers in the control fish were normal (Fig. 4.1) as previously described (Tsai *et al.*, 199X). Most of the pathological effects were observed in the liver, but a few neoplasms were found in the intestine, pancreas, and body wall. Lesions in the liver included cytotoxicity, biliary hyperplasia, foci of cellular alteration, and both benign and malignant neoplasms. These are enumerated in Table 4.2. Cytotoxicity was only

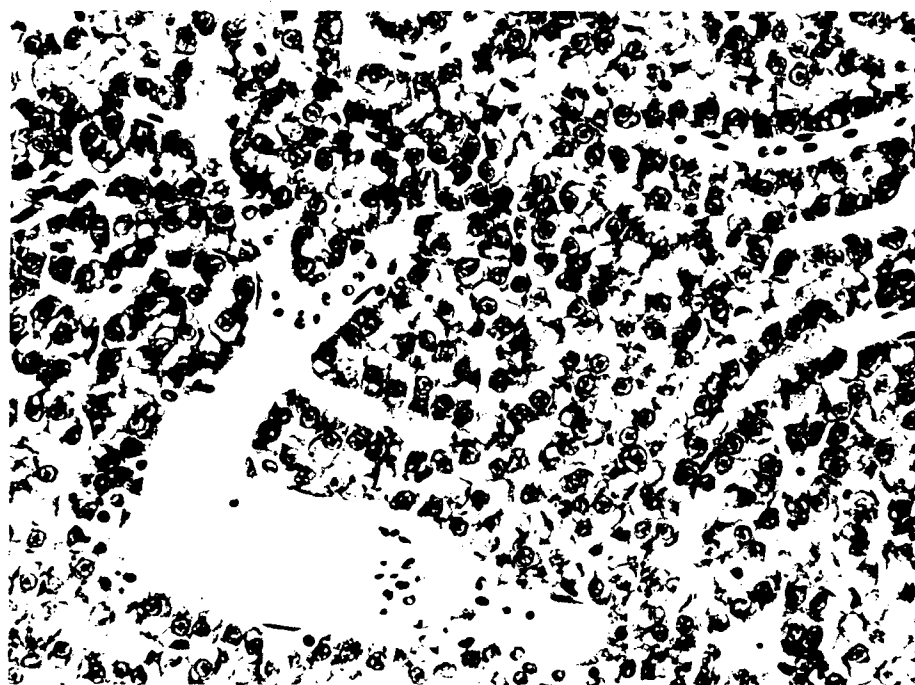


Fig. 4.1. Normal liver from control zebrafish. The two-cell-wide nature of the hepatic tubule cut longitudinally is clearly evident. Note also the uniform size and shape of hepatocyte nuclei, prominent single nucleolus, and moderate level of glycogen vacuolation. H&E, X544.

Table 4.2. Neoplasia and associated lesions in zebrafish taken 24 wk after the start of dietary exposure to MAM-Ac^a.

Lesion types	Lot	Total No. of tumors observed at each dose of MAM-Ac (ppm) ^b			
		0	500	1000	2000
Non-neoplastic lesions					
CT ^c	1	-	-	-	3/79
	2	-	-	-	-
BH	1	-	1/73	-	7/79
	2	-	-	3/82	2/73
Liver lesions - altered foci					
CF	1	-	2/73	-	5/79
	2	-	1/65	3/82	2/73
EF	1	-	-	-	7/79
	2	-	-	-	6/73
BF	1	-	1/73	2/83	2/79
	2	-	1/65	3/82	5/73
Liver neoplasms					
HCA	1	-	-	-	1/79
	2	-	-	-	2/73
HCC	1	-	-	3/83	9/79
	2	-	-	5/82	13/73

Table 4.2 (continued)

CCA	1	-	-	1/80	4/79
	2	-	-	3/82	5/73
CCC	1	-	-	-	8/79
	2	-	1/82	1/80	2/73
MC	1	-	-	-	1/79
	2	-	-	-	-
HB	1	-	-	-	-
	2	-	-	-	1/73
Other types of neoplasia					
AC	1	-	-	-	1/79
(intestine)	2	-	-	-	-
LS	1	-	-	-	2/79
(intestine)	2	-	-	-	-
FS (body	1	-	-	-	1/79
wall)	2	-	-	-	-
AC	1	-	-	-	1/79
(pancreas)	2	-	-	-	-

*Fish were fed MAM-Ac diet for 12 wk then control diet for an additional 12 wk

^bSome fish had more than one lesion

^cAbbreviations used: CT - cytotoxicity, BH - biliary hyperplasia, CF - clear cell foci, EF - eosinophilic foci, BF - basophilic foci, HCA - hepatocellular adenoma, HCC - hepatocellular carcinoma, CCA - cholangiocellular adenoma, CCC - cholangiocellular carcinoma, MC - mixed carcinoma, HB - hepatoblastoma, AC - adenocarcinoma (intestine), LS - leiomyosarcoma (intestine), FS - fibrosarcoma (body wall), AC - adenocarcinoma (pancreas)

observed in fish fed the highest dose of MAM-Ac, and then in only a few fish. It consisted of cellular swelling (megalocytosis) and focal areas of necrosis. Biliary hyperplasia was seen in all groups but primarily in the highest dose group. Profiles of immature bile ducts occurred in higher numbers than are normally seen along biliary tracts.

As with other exposures to hepatocarcinogens, foci of altered hepatocytes increased in a dose-responsive manner. These consisted of eosinophilic, basophilic, and clear cell variants. The eosinophilic focus was most common. These foci had enlarged cells with little or no glycogen and a distinctly granular eosinophilic cytoplasm. The organization of these cells into hepatic tubules was normal (Fig. 4.2). Basophilic foci appeared essentially normal except for the distinct basophilic staining and lack of glycogen in the cells (Fig. 4.3). Clear cell foci were composed of enlarged cells that were completely vacuolated by what appears to be glycogen. Nuclei were compressed and in either centric or eccentric positions (Fig. 4.2).

Few hepatocellular adenomas were observed. One from the high dose level group was eosinophilic and similar to the eosinophilic foci only larger. The cells occupied well defined hepatic tubules, were larger than normal, and had distinct pink granular cytoplasm and few glycogen vacuoles (Figs. 4.4 & 4.5). Hepatocellular carcinomas (HCC) were the most abundant neoplasms observed with this treatment. Figs. 4.6 & 4.7 present a large HCC with distinct basophilia, slightly enlarged, somewhat irregular nuclei, frequent mitotic figures, tubules with increased numbers of cells in longitudinal profile, and invasiveness to normal liver. One fish in the 2000 ppm group had a neoplasm that was tentatively identified as a hepatoblastoma (Figs.

4.8 & 4.9). It occurred adjacent to a HCC, but its cells were more poorly differentiated or embryonal, mitotic figures were numerous, and its apparent aggressive growth pattern had outstripped its blood supply, leading to a large central necrotic zone. Hepatic tumors of similar appearance in mummichog (Vogelbein *et al.*, 1990) and rainbow trout (Bailey *et al.*, 1996), have been assigned this tentative classification.

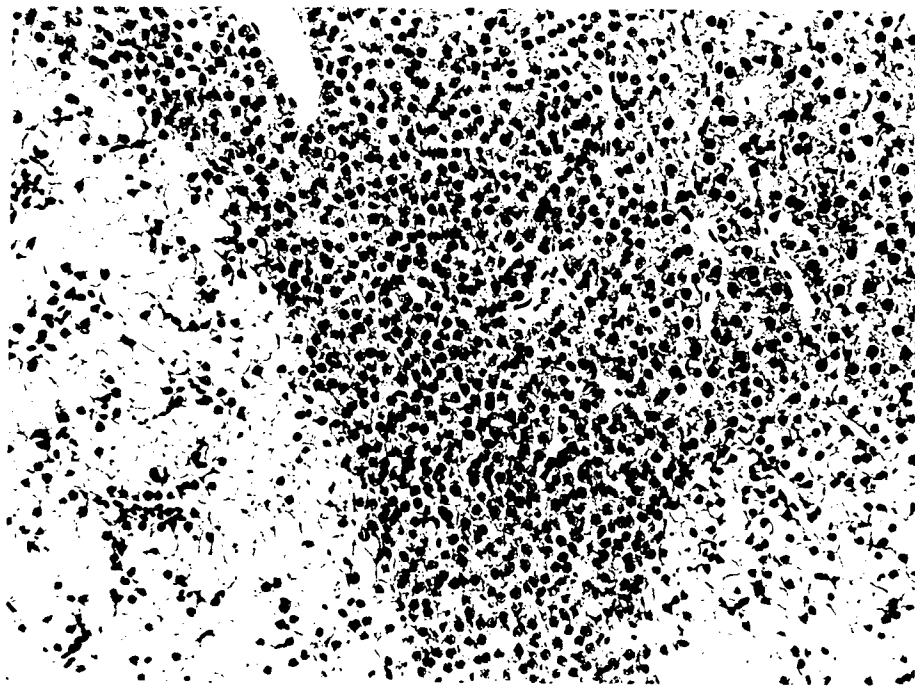


Fig. 4.2. An eosinophilic focus (upper right) and two clear cell foci in the liver of a zebrafish exposed to 75 ppm MAM-Ac in a fry water bath. An area of normal liver occurs between the foci. H&E, X340.

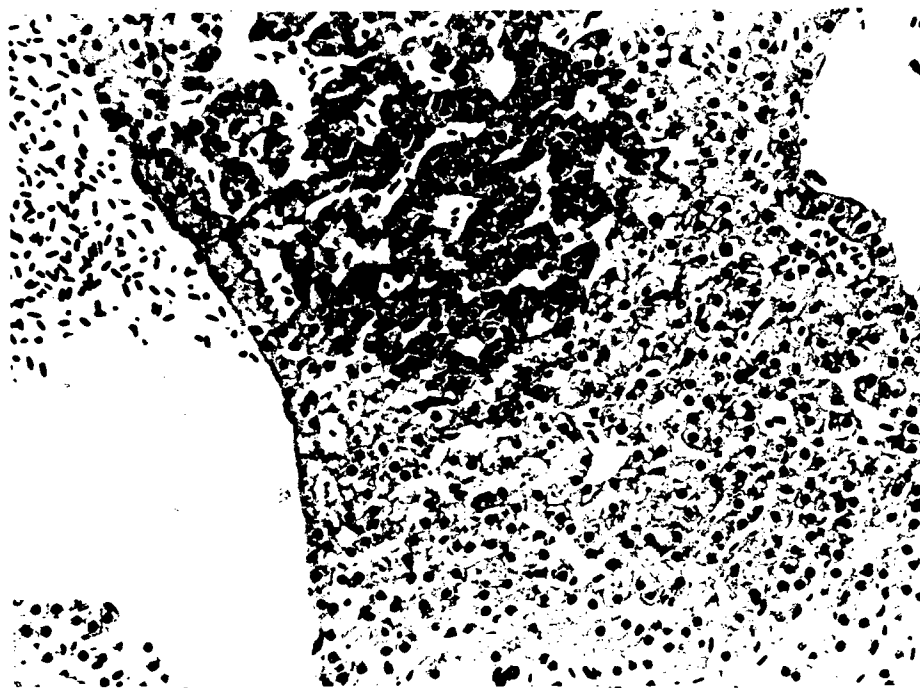


Fig. 4.3. A basophilic focus in the liver of a zebrafish exposed to 2000 ppm dietary MAM-Ac. Hepatic tubular structure is normal, but staining is distinctly basophilic. H&E, X340.



Fig. 4.4. An eosinophilic adenoma in a zebrafish exposed to 75 ppm MAM-Ac in a fry water bath. Normal liver is present at the far left. H&E, X136.

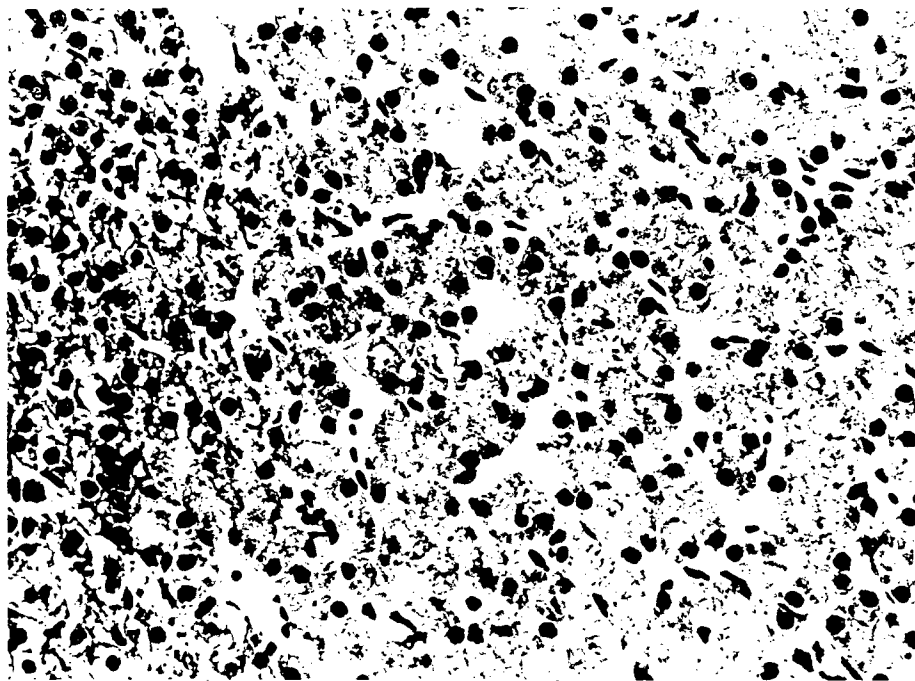


Fig. 4.5. A higher magnification of Fig. 4, showing the enlarged hepatocytes, granular cytoplasm, and reduced glycogen compared to the normal tissue on the left. H&E, X544.

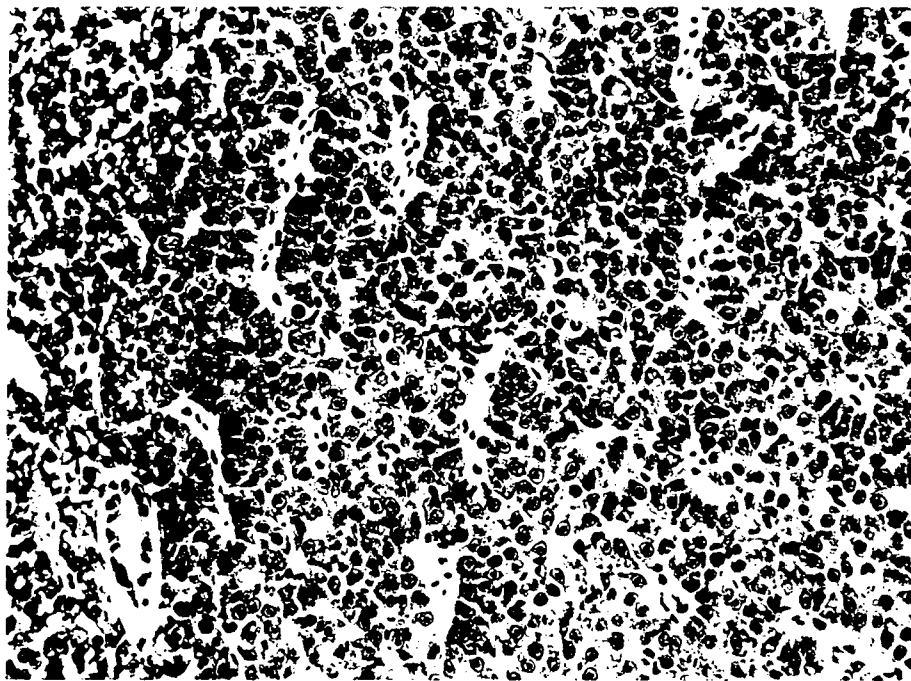


Fig. 4.6. A large hepatocellular carcinoma in a zebrafish exposed to 2000 ppm dietary MAM-Ac. Hepatic tubules are multiple cells wide between adjacent sinusoids, cells are deeply basophilic, devoid of glycogen and mitotic figures are common. Normal liver is on the left H&E, X340.

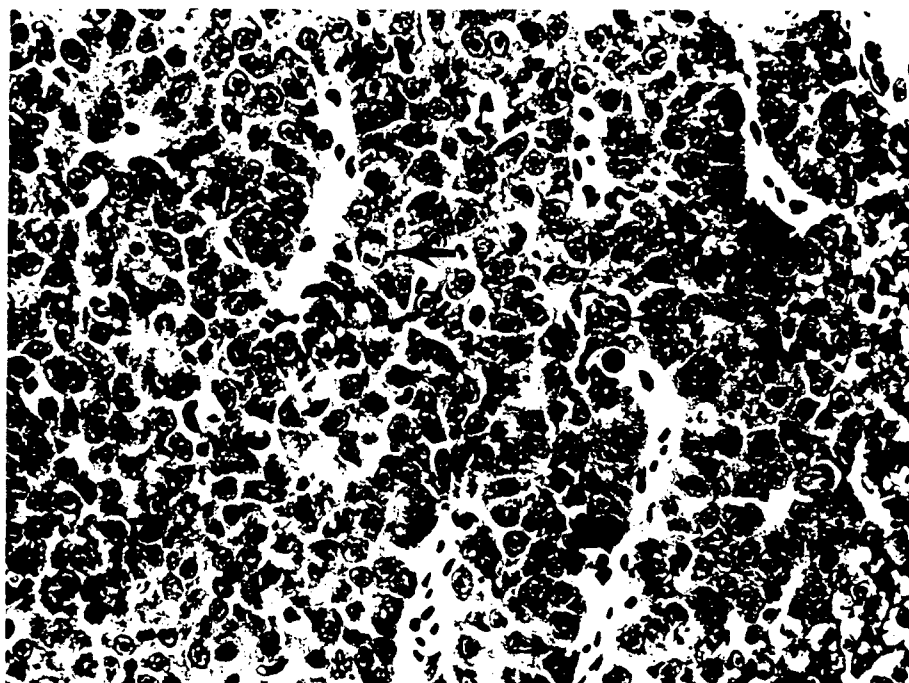


Fig. 4.7. Detail of Fig. 6, mitotic figures are easily seen (arrows) and the broad hepatic tubules are clearly evident. H&E, X544.

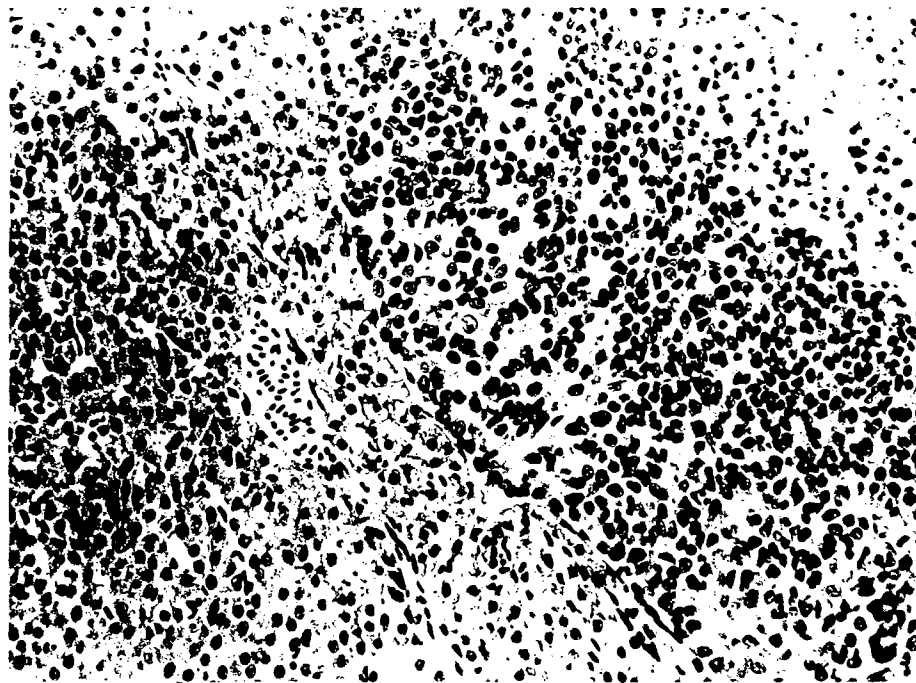


Fig. 4.8. A portion of the liver from a zebrafish fed 2000 ppm MAM-Ac. On the left is a hepatocellular carcinoma, next to it is a band of non-neoplastic liver, and on the upper right is a unique neoplastic mass with some features resembling a hepatoblastoma. At the far upper right is a large necrotic area which is part of the putative hepatoblastoma. H&E, X340.

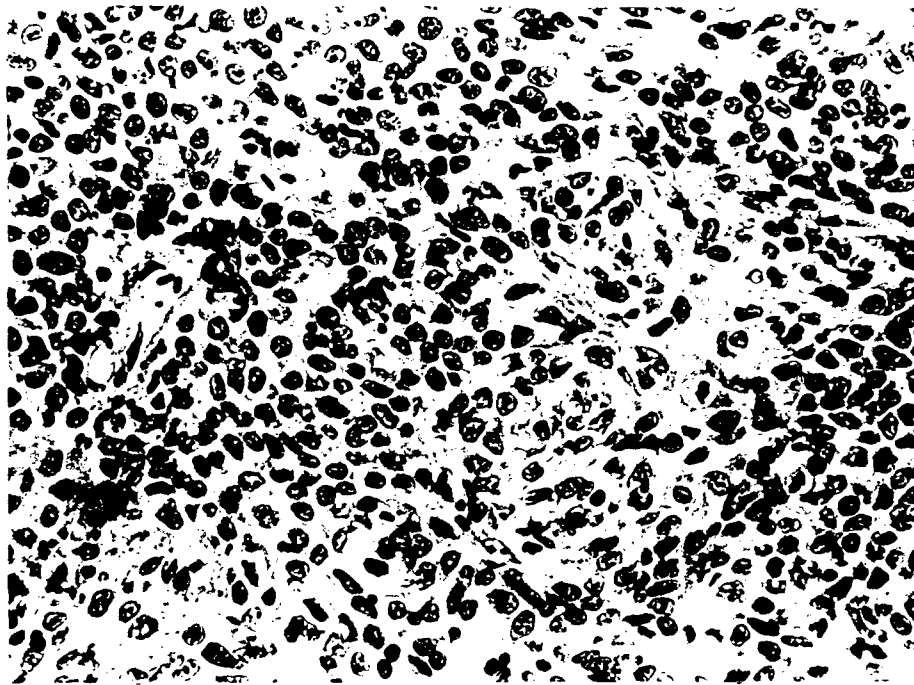


Fig. 4.9. Greater detail of Fig. 4.8. The cells are less differentiated, mitoses are numerous and liver architecture is completely disrupted. H&E, X544.

Biliary neoplasms included both benign, small, encapsulated cholangiocellular adenomas (Fig. 4.10), and malignant, large, invasive cholangiocellular carcinomas (Figs. 4.11 & 4.12). One large neoplasm from the 2000 ppm exposed groups was clearly a mixed carcinoma, with both hepatocellular and cholangiocellular components intermingled (Fig. 4.13).

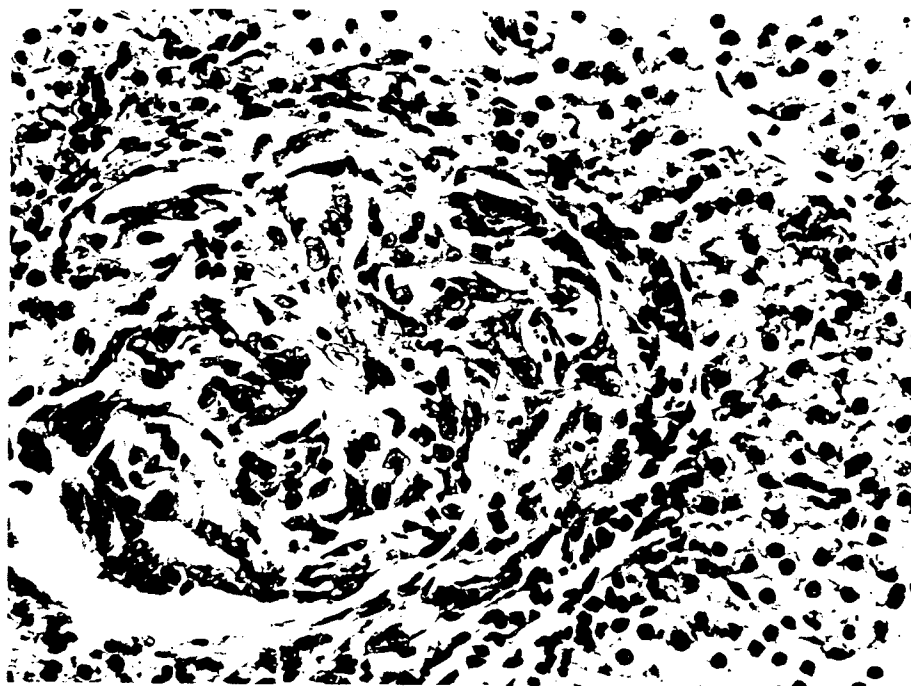


Fig. 4.10. A small encapsulated cholangiocellular adenoma in a zebrafish fed 1000 ppm MAM-Ac. H&E, X544.

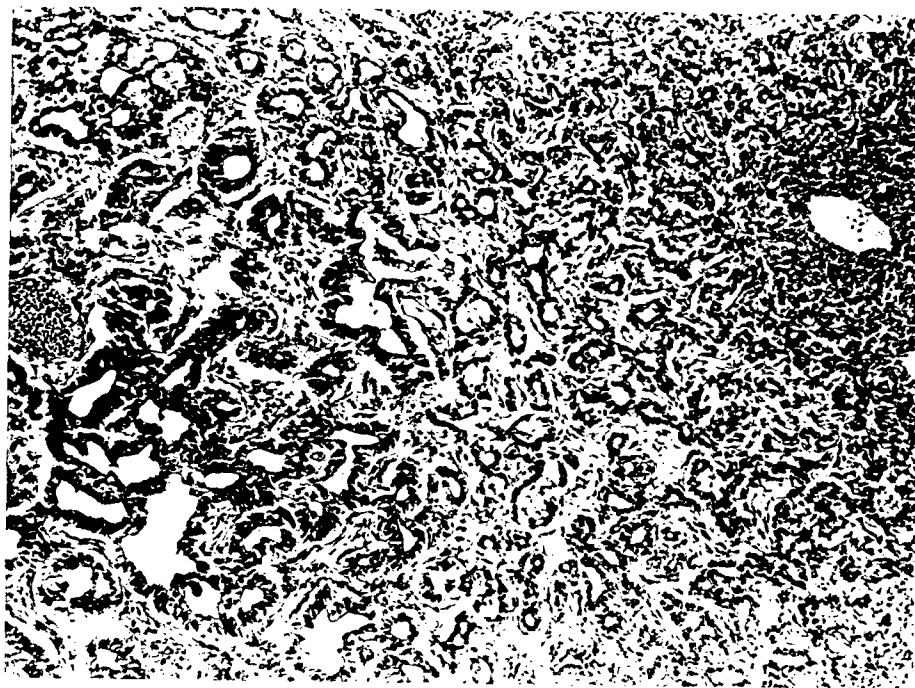


Fig. 4.11. A large cholangiocellular carcinoma from a zebrafish exposed to the 75 ppm MAM-Ac fry water bath. Note the branching biliary ducts and invasion of liver at upper right. H&E, X136.

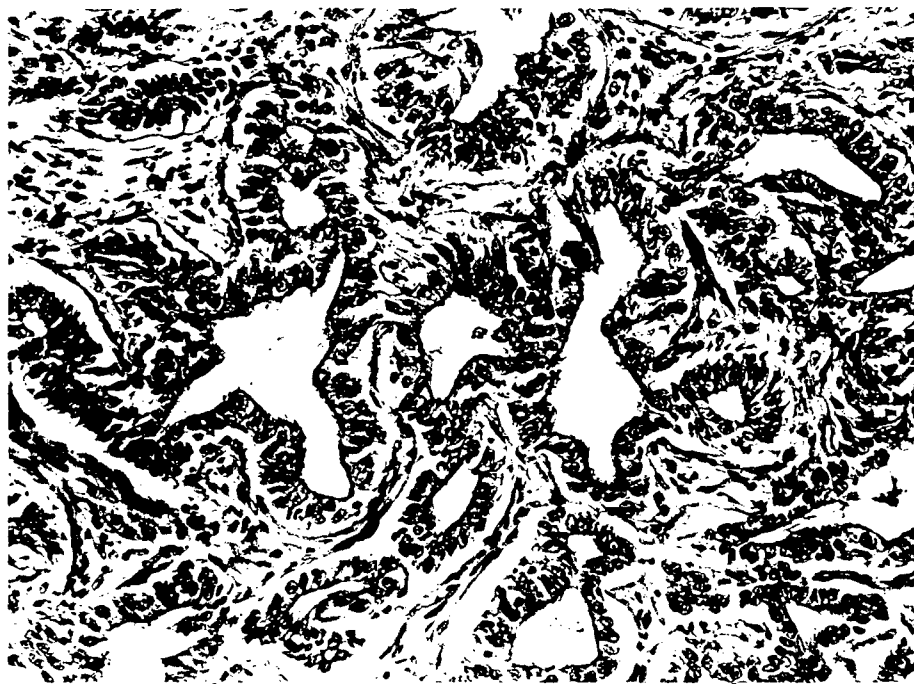


Fig. 4.12. A higher magnification photograph of the carcinoma in Fig. 4.11. H&E, X340.

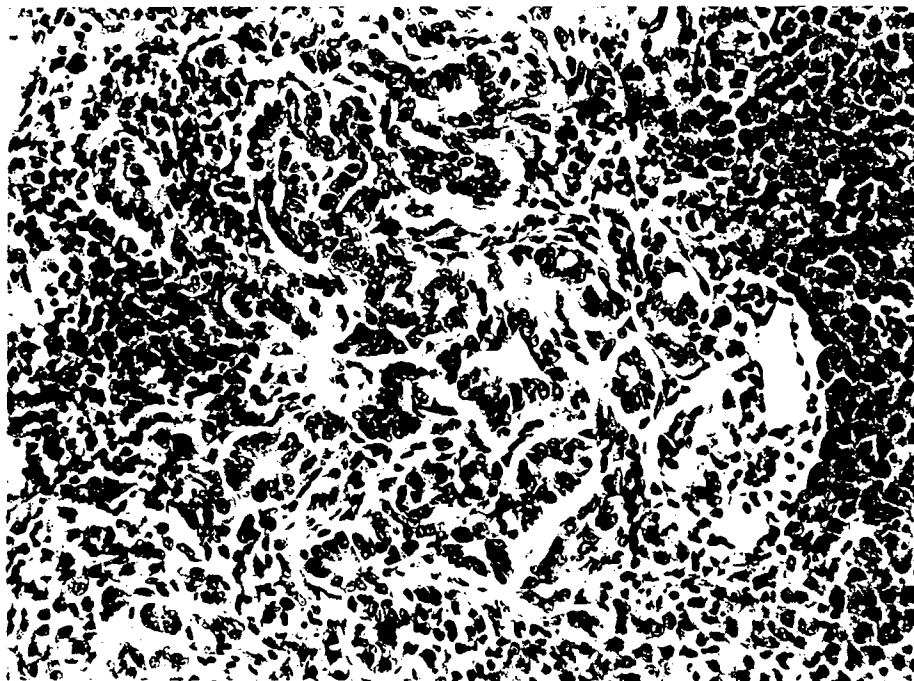


Fig. 4.13. A mixed carcinoma having hepatocellular (far right) and biliary components intermixed. The individual parts are similar those in either hepatocellular or cholangiocellular carcinomas. H&E, X340.

Other, nonhepatic neoplasms originating in the groups fed the diet containing 2000 ppm MAM-Ac included, 1) a fibrosarcoma, arising from the dermis and invading through the muscle wall into the peritoneal cavity (Figs. 4.14 & 4.15). The tumor was made up of whorls and bundles of fibroblasts, with plump nuclei and occasional mitotic figures, 2) a leiomyosarcoma originating in the tunica muscularis of the intestine. This tumor was large, invasive and had interlacing bundles of smooth muscle cells, with large nuclei and occasional mitotic figures (Fig. 4.16), 3) a ductal adenocarcinoma originating in the pancreas (Fig. 4.17). Although well differentiated, this tumor showed tendencies to invade the intestine and liver, and had replaced most of the pancreatic acinar tissue, and 4) an adenocarcinoma of the intestinal mucosa, with poorly differentiated, basophilic nests of cells invading the deeper levels of the intestinal wall (Fig. 4.18).



Fig. 4.14. A fibrosarcoma arising from the dermis of the skin, penetrating through the muscle wall and into the peritoneal cavity. On the far right is the liver. H&E, X136.



Fig. 4.15. Higher magnification of the tumor mass in Fig. 4.14, showing whorls and bundles of fibroblasts. H&E, X340.

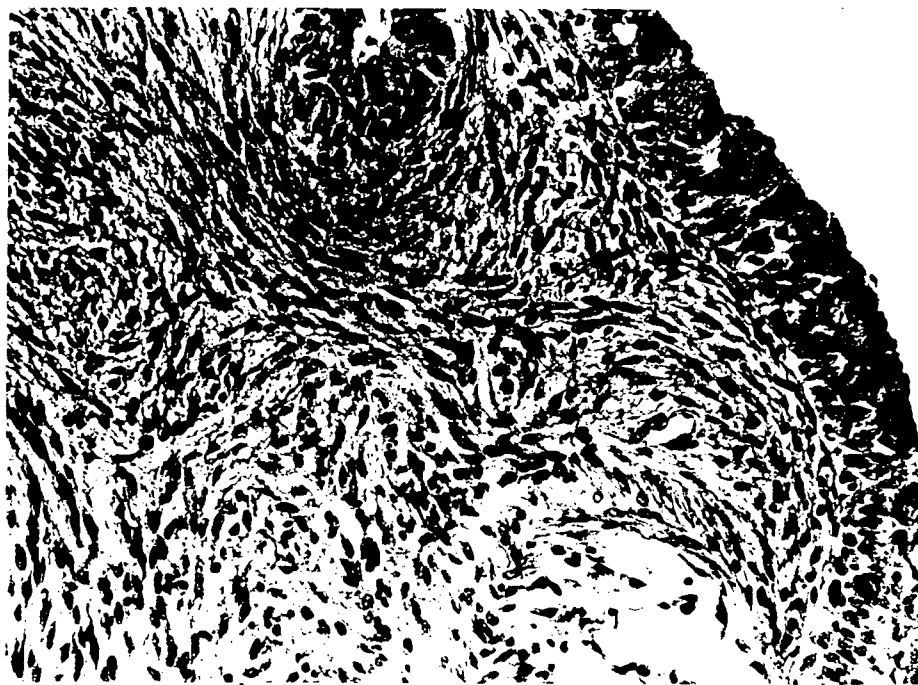


Fig. 4.16. A leiomyosarcoma arising in the tunica muscularis of the intestinal wall, with mucosa at upper right. Zebrafish was fed 2000 ppm MAM-Ac. Bundles of fibroblasts, having relatively large nuclei, have replaced some of the mucosa and invaded and destroyed the entire intestinal wall. H&E, X340.



Fig. 4.17. Pancreatic ductal adenocarcinoma in a zebrafish fed 2000 ppm MAM-Ac. The pancreas has been replaced by the neoplastic ducts (central region), Normal intestine is on the right, liver at lower left. H&E, X136.

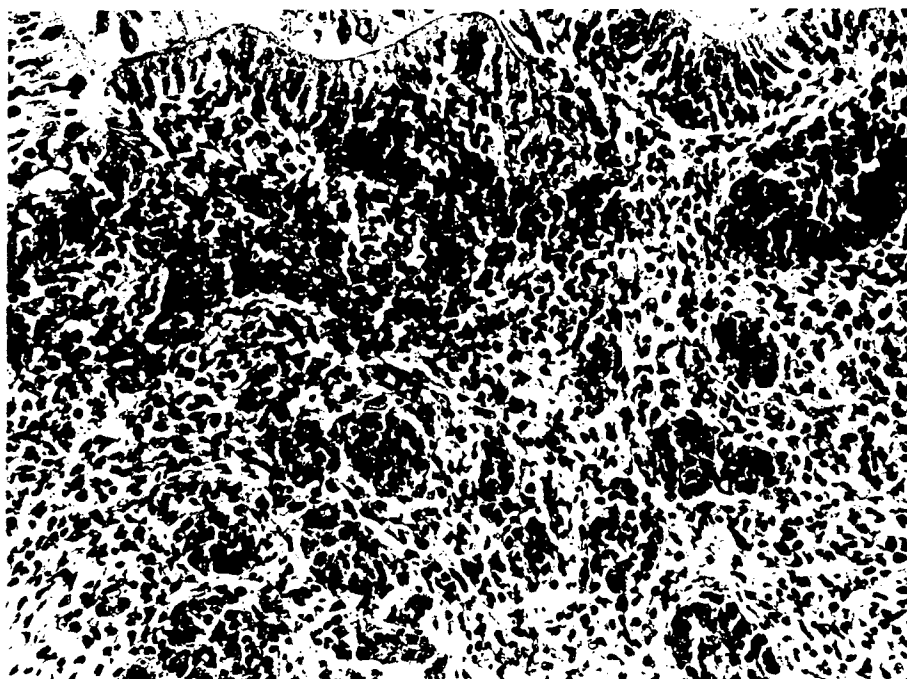


Fig. 4.18. An intestinal adenocarcinoma in a zebrafish exposed to 2000 ppm dietary MAM-AC. Poorly differentiated nests of neoplastic cells are invading downward into the intestinal wall. H&E, X340.

Dietary Exposure - medaka

The data comparing the response of zebrafish and medaka to dietary MAM-Ac at 1000 and 2000 ppm is presented in Table 4.3. As shown, the responses of the two species were very similar. There was no evidence of a difference between species of fish ($p > 0.5$, 1 df) or interaction lack of fit ($p > 0.5$, 1 df). The incidence of liver neoplasms was slightly higher in medaka than zebrafish, but medaka did not develop neoplasms in other tissues as did the zebrafish. A lesion that was commonly seen in medaka but not in zebrafish was spongiosis hepatitis (Fig. 4.19). The status of this lesion is still controversial, but it is interesting that it occurs regularly in carcinogenesis studies with medaka, and to date we have not observed it in zebrafish. The other hepatic neoplasms in medaka have been illustrated numerous times in the literature and photographs of these lesions will not be included in this paper.

Table 4.3. Carcinogenic response of zebrafish and medaka to dietary MAM-Ac^{ab}

Species	MAM-Ac dose ppm	Mortality %	Neoplastic response	
			Inc. ^c	% ^f
Zebrafish	0	9	0/163	0
Zebrafish	1000	8	13/165	8
Zebrafish	2000	16	39/152	26
Medaka	0	0	0/100	0
Medaka	1000	5	6/95 ^d	6
Medaka	2000	28	20/72 ^e	28

^aFish were fed MAM-Ac diet for 12 wk then control diet for an additional 12 wk

^bData for zebrafish exposed to 1000 and 2000 ppm MAM-Ac were pooled from Table 4.1

^cNo. of tumor bearing fish/total No. of fish

^dTumors were diagnosed as follows: three cholangiocellular carcinomas, two hepatocellular carcinomas, three hepatocellular adenomas

^eTumors were diagnosed as follows: one hepatocellular adenoma, one cholangiocellular adenoma, seven hepatocellular carcinomas, nine cholangiocellular carcinomas, and two mixed carcinomas

^fThere was a highly significant difference between the two MAM-Ac doses (0.0001, df) but no evidence of a difference between the two species ($p > 0.5$, 1df)

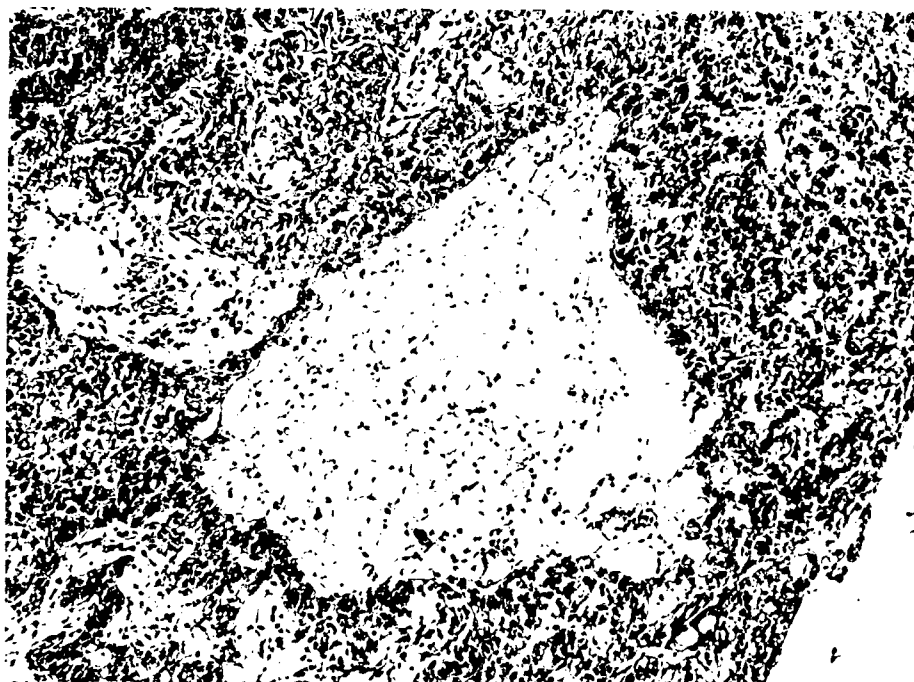


Fig. 4.19. A spongiosis hepatitis lesion from a medaka fed the 2000 ppm dose of MAM-Ac. H&E, X136.

Fry Water Bath Exposure

The results of this part of the study are included in Tables 4.4 and 4.5. Both the mortalities and neoplastic response were dose related, but the overall response was low, only 12 and 14% at the two highest doses, respectively. Since neoplasms were observed only at the three highest doses, and all these doses produced treatment related mortalities, there appears to be a very narrow range between lethality and carcinogenicity with this exposure route. The spectrum of lesions resulting from this route of exposure was very similar to that seen with dietary exposure, and the liver was once again the primary target organ. Since the neoplasms were essentially the same as with dietary exposure, photographs of these lesions will not be included, with the exception of an osteosarcoma of the gill in a fish exposed to 75 ppm MAM-Ac (Fig. 4.20). This photo shows the proliferation of chondrocytes, differentiation into bone, and invasion and disruption of the gill arch.

Table 4.4. Carcinogenic response of 21-day post-hatch zebrafish fry exposed to static water solutions of MAM-Ac^a

Lot	MAM-Ac dose ppm	Mortality %	Neoplastic response	
			Inc. ^b	%
1	0	0	0/100	0
2	6.25	0	0/100	0
3	12.5	0	0/100	0
4	25	0	0/100	0
5	50	15	4/85	5
6	75	26	9/74	12
7	100	35	9/65	14

^aZebrafish fry were exposed to MAM-Ac solutions for 2 hr and terminated 12 mo later

^bNo. of tumor bearing fish/total No. of fish

Table 4.5. Numbers of neoplastic and associated lesions of zebrafish terminated 1 yr after a 2 hr exposure of fry to MAM-Ac

Lesion types	Total No. of tumors observed at different doses of MAM-Ac (ppm) ^a						
	0	6.25	12.5	25	50	75	100
Non-neoplastic lesions							
CT ^b	0/100	0/100	0/100	0/100	0/85	2/74	10/65
BH	0/100	0/100	0/100	0/100	0/85	1/74	2/65
Liver lesions - Foci of altered cells							
BF	0/100	0/100	0/100	0/100	3/85	1/74	1/65
EF	0/100	0/100	0/100	0/100	0/85	4/74	3/65
CF	0/100	0/100	1/100	0/100	0/85	4/74	1/65
Liver Neoplasms							
HCA	0/100	0/100	0/100	0/100	1/85	5/74	4/65
HCC	0/100	0/100	1/100	0/100	0/85	4/74	5/65
CCA	0/100	0/100	0/100	0/100	1/85	1/74	1/65
CCC	0/100	0/100	0/100	0/100	0/85	0/74	3/65
Other tissues							
OS (gill)	0/100	0/100	0/100	0/100	0/85	1/74	0/65

^aSome fish had more than one lesion

^bAbbreviations used: CT - cytotoxicity, BH - biliary hyperplasia, BF - basophilic foci, EF - eosinophilic foci, CF - clear cell foci, HCA - hepatocellular adenoma, HCC - hepatocellular carcinoma, CCA - cholangiocellular adenoma, CCC - cholangiocellular carcinoma, OS - osteosarcoma



Fig. 4.20. An osteosarcoma of the gill in a zebrafish exposed to 50 ppm MAM-Ac in a fry water bath. Note the proliferation of cartilage, differentiation into bone, and the disruption of the gill arch. H&E, X340

Embryo Water Bath Exposure

Zebrafish exposed to MAM-Ac as embryos suffered high mortalities particularly in the early stages of the experiment (Table 4.6). This occurred in the controls, but there was also an obvious treatment effect as well. When an experiment is started with embryos, and losses are based on the initial number of embryos, mortalities run higher than if the initial number is based on fry or adults that have survived the more vulnerable stages of hatching, swimup, and the onset of feeding. At termination, there was no evidence of tissue damage that could have explained the earlier mortalities. Although there were few survivors at the highest dose, tumor incidences were the highest (11/20, and 12/17) of all the exposure routes tested. Among those that survived, there were highly significant differences in tumor incidence between treatments ($p < 0.0001$, 2 df). Over the three MAM-Ac doses (10 to 50 ppm) there was a significant linear dose response ($p < 0.0001$, logistic regression, 1 df) with no evidence of lack of fit to linear ($p > 0.5$, 1 df). Liver lesions and neoplasia were similar to those observed in the other exposures to MAM-Ac (Table 4.7).

Of particular interest, was the wide variety of neoplasms that developed in extrahepatic tissues. Some of these were also epithelial tumors, but most were mesenchymal or neural in origin. In all cases, only single tumors in single fish resulted from the treatment with this direct-acting carcinogen. Thus, although these neoplasms were clearly the result of MAM-Ac exposure, no single extrahepatic tissue or organ was more susceptible to MAM-Ac than another. The tumors observed were as follows. Two fish developed seminomas in the testis in the groups exposed to 10

ppm MAM-Ac. Four separate fish in the 25 ppm groups developed the following tumors: A seminoma in the testis, a leiomyosarcoma in the intestine, a chondrosarcoma in the gill, and an adenoma in the exocrine pancreas. In the 37 survivors of the 50 ppm MAM-Ac embryo treatment, there were the following neoplasms: one ductal adenoma in the pancreas, one osteochondroma in the scleral layer of the eye (Fig. 4.21), one neurofibroma in the skeletal muscle of the trunk region, one hemangioma in the pharynx, one hemangioma in the ovary, one hemangioma on the tail (Fig. 4.22), one hemangioma in the gill, one

Table 4.6. Carcinogenic response of zebrafish, exposed to MAM-Ac for 12 hr as 72-hr embryos, and terminated 12 mo later

Lot	MAM-Ac dose ppm	Mortality %	Neoplastic response Inc. ^a	% ^b
1	0	35	0/97	0
2	0	52	0/72	0
1	10	51	4/73	5
2	10	55	2/67	3
1	25	62	8/57	14
2	25	77	4/35	11
1	50	87	11/20	55
2	50	89	12/17	71

^aNo. of fish with tumors/total No. of fish

^bThere were significant differences in tumor incidence between treatments (p<0.0001, 2 df)

Table 4.7. Numbers of neoplastic and associated lesions in zebrafish liver exposed to MAM-Ac as embryos

Lesion types ^a	Lot	Total No. of tumors observed at different doses of MAM-Ac (ppm)			
		0	10	25	50
Non-neoplastic lesions					
ME	1	0/97	0/73	0/57	0/20
	2	0/72	0/67	1/35	0/17
BH	1	1/97	0/73	2/57	6/20
	2	3/72	2/67	3/35	4/17
Foci of altered hepatocytes					
CF	1	0/97	1/73	1/57	1/20
	2	0/72	0/67	0/35	1/17
EF	1	1/97	0/73	7/57	1/20
	2	0/72	0/67	1/35	1/17
BF	1	0/97	1/73	0/57	0/20
	2	0/72	0/67	0/35	0/17
Neoplasms					
HCA	1	0/97	3/73	4/57	5/20
	2	0/72	1/67	1/35	3/17
HCC	1	0/97	0/73	1/57	2/20
	2	0/72	0/67	0/35	5/17

Table 4.7 (continued)

CCA	1	0/97	0/73	2/57	1/20
	2	0/72	0/67	0/35	1/17
CCC	1	0/97	0/73	0/57	1/20
	2	0/72	0/67	0/35	1/17
MA	1	0/97	0/73	0/57	1/20
	2	0/72	0/67	0/35	0/17
MC	1	0/97	0/73	0/57	0/20
	2	0/72	0/67	0/35	1/17

*Abbreviations used: ME - megalocytosis, BH - biliary hyperplasia, CF - clear cell foci, EF - eosinophilic foci, BF - basophilic foci, HCA - hepatocellular adenoma, HCC - hepatocellular carcinoma, CCA - cholangiocellular adenoma, CCC - cholangiocellular carcinoma, MA - mixed adenoma, MC - mixed carcinoma

medulloepithelioma in the retina of the eye (Fig. 4.23), one renal adenoma (Fig. 4.24), one neurofibrosarcoma of the spine (Fig. 4.25), one neuroblastoma in cranial area (Figs. 4.26 & 4.27), and one rhabdomyoma in the heart (Figs. 4.28 & 4.29). All these neoplasms were single lesions in separate fish.



Fig. 4.21. Osteochondroma in the scleral layer of the eye. Mass is comprised of well-differentiated cartilage and bone. Embryonic treatment with 50 ppm MAM-Ac for 12 hr. H & E, X136.

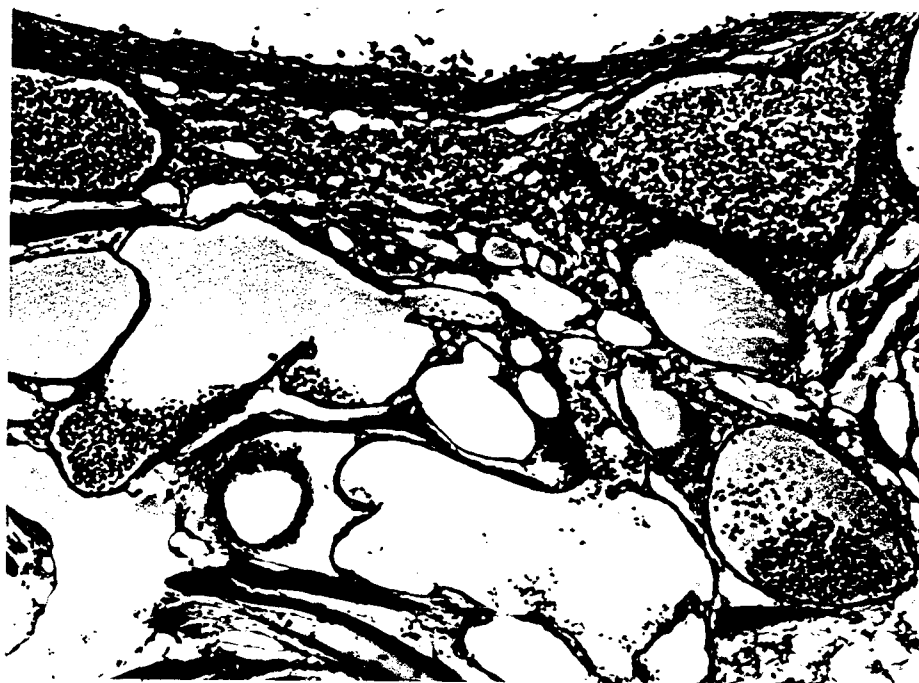


Fig. 4.22. Cavernous hemangioma in the caudal peduncle. Multicystic subcutaneous mass contains variably-sized, ovoid, blood-filled spaces lined by well-differentiated endothelium. Hemorrhage, hemosiderin-laden macrophages and associated fibrosis surround the largest cyst. Embryonic exposure to 50 ppm MAM-Ac for 12 hr. Moribund fish sacrificed at 7 mo post-treatment. H & E, X136.

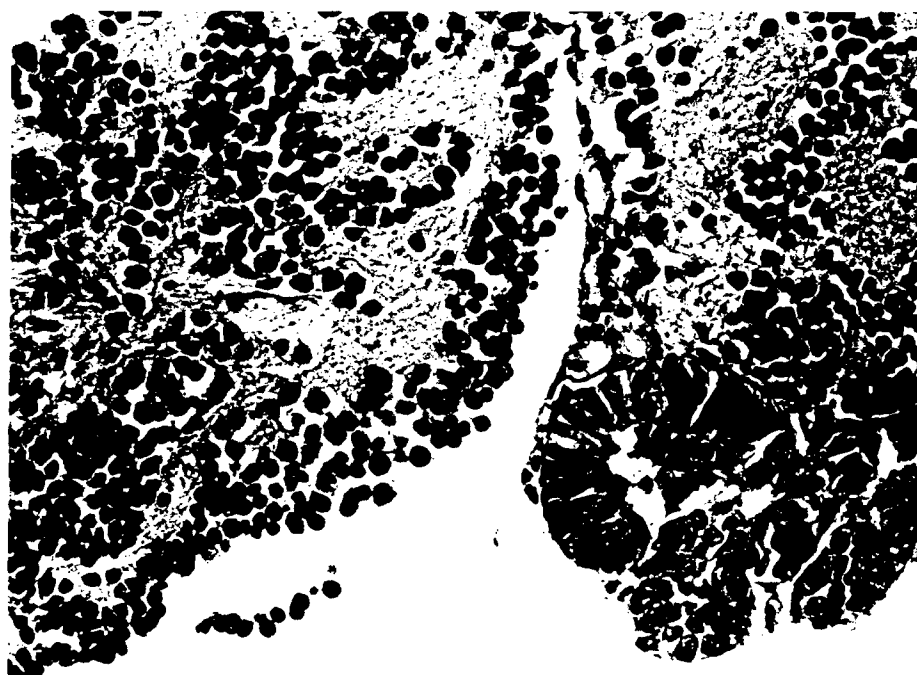


Fig. 4.23. Medulloepithelioma in the retina. Mass consists predominately of well-differentiated neuroepithelial cells in a haphazard arrangement set in abundant neuropil. A smaller component of the neoplasm is comprised of cords of less well-differentiated columnar neuroepithelium with basally oriented nuclei. Embryonic exposure to 50 ppm MAM-Ac for 12 hr. H & E, X544.

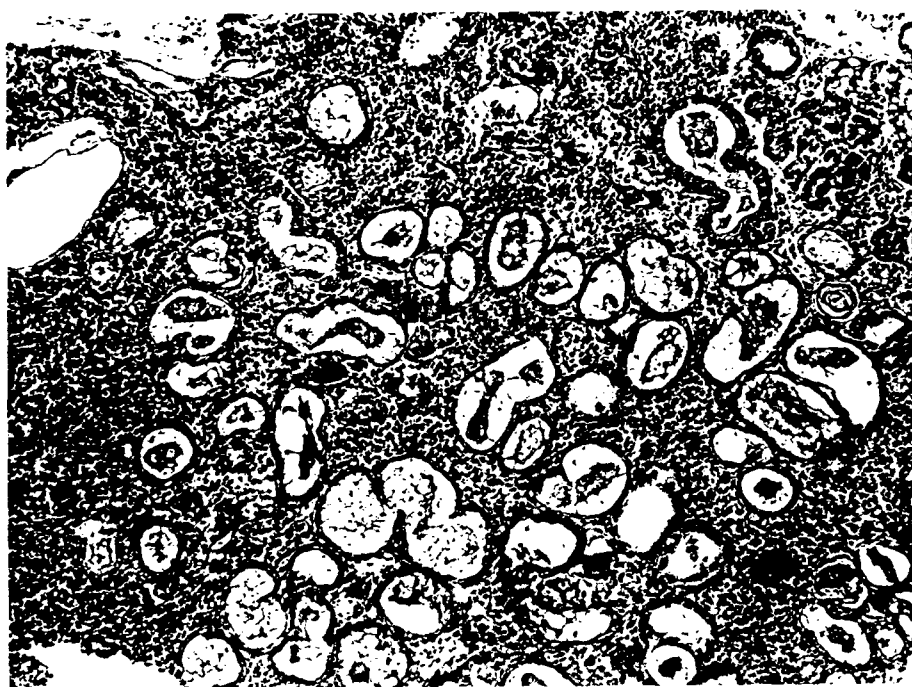


Fig. 4.24. Renal tubular adenoma in trunk kidney. Mass is formed by well-differentiated tubules composed of cuboidal epithelium. Most tubules are moderately dilated and filled with lightly basophilic fluid. Embryonic exposure to 50 ppm MAM-Ac for 12 hr. H & E, X136.



Fig. 4.25. Neurofibrosarcoma of the spine. Mass surrounds spine and multifocally invades vertebrae. Neoplasm consists of spindloid to stellate cells arranged in a storiform pattern, set in moderate amounts of neuropil. Verocay body formation and nuclear palisading is evident. Embryonic exposure to 50 ppm MAM-Ac for 12 hr. Moribund fish sacrificed at 7 mo post-treatment. H & E, X136.



Fig. 4.26. Neuroblastoma of skull. Extensive mass grossly distorting skull, surrounding brain and infiltrating optic nerve and anterior vertebrae. Embryo exposure to 50 ppm MAM-Ac for 12 hr. Moribund fish sacrificed at 7 mo post-treatment. H & E, X34.

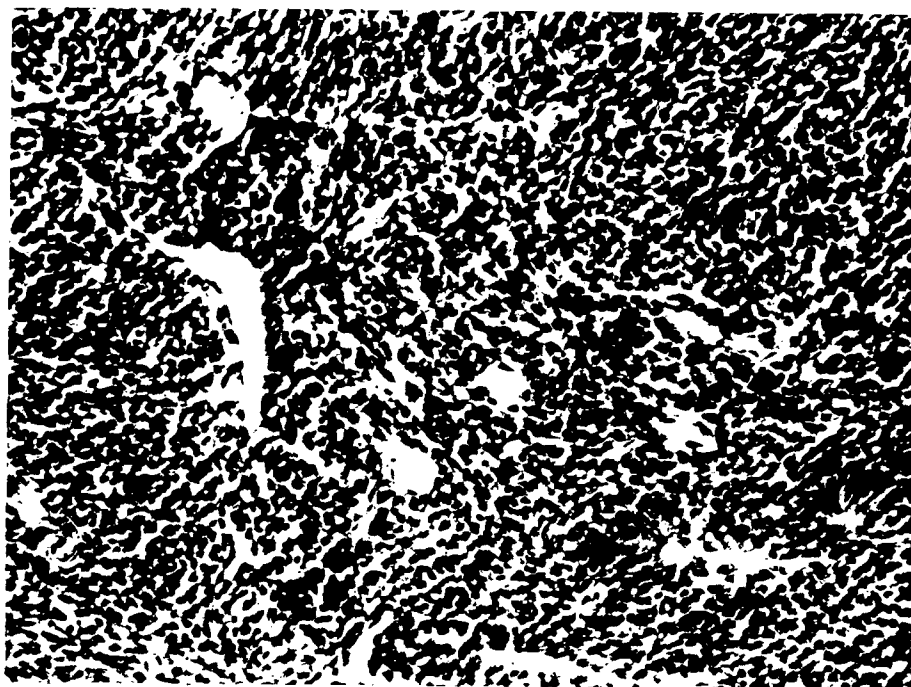


Fig. 4.27. Higher magnification of the neuroblastoma of Fig. 4.26. Neoplasm consists of tightly packed, uniform round neuroblasts with negligible cytoplasm, set in scant neuropil. Definitive neuroepithelial rosettes are not evident. H & E, X340.



Fig. 4.28. Rhabdomyoma in the ventricle of the heart, surrounding the atrio-ventricular valve. Mass consists of plump, densely-packed ovoid myocytes with central oval nuclei and abundant granular to vacuolated cytoplasm lacking clear striations. Embryonic exposure to 50 ppm MAM-Ac for 12 hr. H & E, X136.

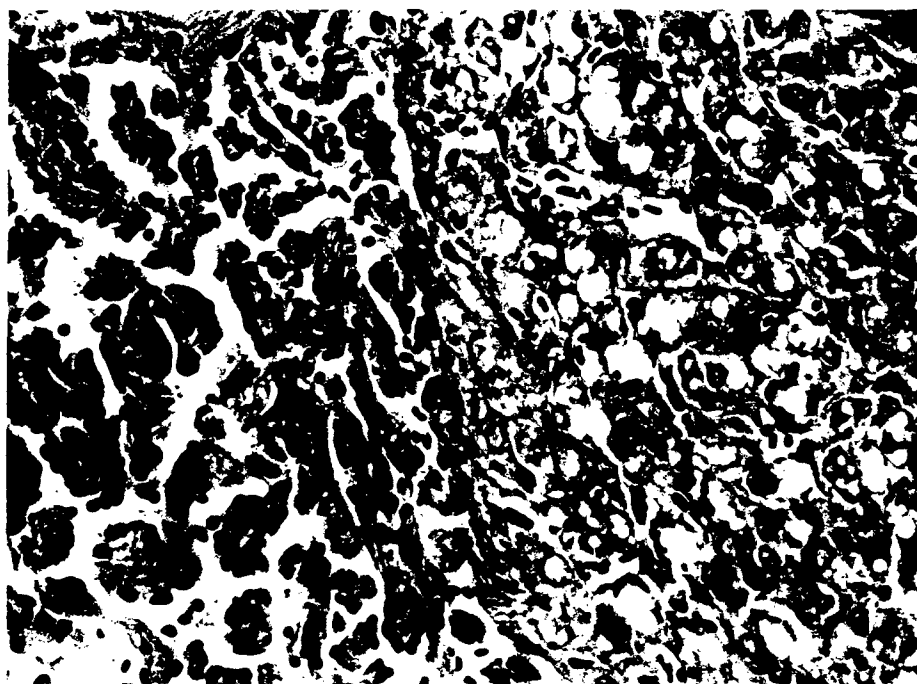


Fig. 4.29. Higher magnification of the rhabdomyoma of Fig. 4.28. The transition between rhabdomyoma (right) and normal ventricular tissue (left) is demonstrated. H & E, X544.

DISCUSSION

Previous experiments with zebrafish at our laboratory have shown a trend towards relative insensitivity to the carcinogenicity of nitrosamines and aflatoxin B₁ (Tsai *et al.*, 199X; Tsai and Hendricks, 199X), however, we had not conducted any side-by-side comparisons with other species. In this study, dietary exposure of zebrafish and medaka to the same MAM-Ac-containing diets revealed an almost identical carcinogenic response in the two species. Khudoley (1984) compared zebrafish and guppies with respect to nitrosamine carcinogenicity and concluded that zebrafish were more sensitive than guppies. Hawkins *et al.* (1985) compared seven species of aquarium fish and concluded that the medaka and guppy were both desirable models but did not make any quantitative comparisons. Additional species to species comparisons in the same laboratory would be useful for ranking the various species with respect to sensitivity.

Route of exposure can have a pronounced effect on target organ response, especially with a direct-acting carcinogen (Hendricks *et al.*, 1980; Hendricks *et al.*, 1995; Orner *et al.*, 1995). In the present experiment, the effect was not as variable as expected. With all three routes of exposure, the liver was the major target organ, and the response at other organ sites was so low and scattered, there was no secondary target organ. With the embryo water exposure, there was also a relatively narrow dose range in which carcinogenicity occurred without mortality being excessively high.

There was a distinct difference between zebrafish and medaka with respect to

the occurrence of spongiosis hepatitis. This putative neoplasm of perisinusoidal cells or cells of Ito occurs frequently in carcinogenesis studies with medaka (Hinton *et al.*, 1984) and sheepshead minnow (Couch and Courtney, 1987), but we have never observed it in our carcinogenesis studies with zebrafish. It also has never been described in rainbow trout (Hendricks, Unpublished observations).

Although other carcinogens need to be tested with the zebrafish, there are several characteristics of this species that would limit its usefulness as an environmental monitor or even as a laboratory model. First, there is the apparent lack of responsiveness to certain carcinogens. For this reason this species may not detect carcinogenic problems in the environment as well as a more sensitive species might. Second, and probably most important, is its inability to survive at temperate water temperatures, limiting its use to tropical or sub-tropical waters only. We have found that temperatures below 10°C completely immobilize zebrafish, and their activity is greatly reduced between 10 and 15°C. For prolonged periods of exposure, water temperatures would probably need to be 18°C or higher for zebrafish to sustain normal bodily functions. Third, the location of the zebrafish liver, entwined around the intestine, makes the removal of liver tissue for metabolic studies very difficult and time-consuming. On the other hand, this species is easy to rear, produces eggs all year long, and has a very short development time (96 hr). Although zebrafish have proven to be advantageous for other biological research, we believe their negative characteristics outweigh their positive ones for carcinogenesis. Perhaps this is why no one has perpetuated Stanton's early work with zebrafish. These features may have been known but were never discussed.

ACKNOWLEDGEMENTS

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In Vivo Aflatoxin B₁ Metabolism and Hepatic DNA Adduction in Zebrafish (*Danio rerio*). ¹

Claudia M. Troxel², Ashok P. Reddy³, Patricia E. O'Neal⁴, Jerry D. Hendricks^{2,3,5}, and George S. Bailey^{2,3,5,6}.

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² Toxicology Program, Oregon State University

³ Department of Food Science and Technology, Oregon State University

⁴ Genetics Program, Oregon State University

⁵ Marine/Freshwater Biomedical Sciences Center

⁶ To whom correspondence should be addressed at Department of Food Science and Technology, Marine/Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, OR 97331. Telephone: (541) 737-3164. Fax: (541) 737-1877. E-mail: baileyg@bcc.orst.edu

Aflatoxin B₁ Metabolism and DNA Adduction in Zebrafish.

In Vivo Aflatoxin B₁ Metabolism and Hepatic DNA Adduction in Zebrafish (*Danio rerio*). Troxel, C.M., Reddy, A.P., O'Neal, P.E., Hendricks, J.D., and Bailey, G.S. (1996). *Toxicol. Appl. Pharmacol.*

The zebrafish (*Danio rerio*) is assuming prominence in developmental genetics research. By comparison, little is known of tumorigenesis and nothing is known of carcinogen metabolism in this species. This study evaluated the ability of zebrafish to metabolize a well-characterized human carcinogen, aflatoxin B₁ (AFB₁), to phase I and phase II metabolites and assessed hepatic AFB₁-DNA adduction *in vivo*. Fish i.p. injected with 50-400 µg [³H]AFB₁/kg body weight displayed a linear dose-response for hepatic DNA binding at 24 hours. AFB₁-DNA adduct levels showed no statistical difference over the period from 1 to 21 days after injection, suggesting poor adduct repair in this species. DNA binding in female fish was 1.7-fold higher than in males ($p < 0.01$). An *in vitro* AFB₁ metabolism assay verified that zebrafish liver extracts support AFB₁ oxidation to the 8,9-epoxide proximate electrophile ($K_m = 79.0 \pm 16.4 \mu\text{M}$, $V_{\text{max}} = 11.7 \pm 1.4 \text{ pmol/min/mg}$ at 28°C). The excretion of AFB₁ and its metabolites was also examined by HPLC. As is typical of other fish studied, major metabolites excreted were aflatoxicol (AFL) and aflatoxicol-glucuronide (AFL-g), followed by unreacted AFB₁. AFL appeared as early as 5 minutes after injection, whereas AFL-g was a significant metabolite after 18 hours. This study shows that *in vivo* administration of AFB₁ in zebrafish results in moderate adduction of the carcinogen to liver DNA, and that zebrafish have the capacity for both phase I and phase II metabolism of AFB₁. The approximate 4-fold difference between rainbow trout and zebrafish AFB₁-DNA covalent binding index appears insufficient to explain the relative resistance of zebrafish to dietary AFB₁ hepatocarcinogenicity.

INTRODUCTION

Aquarium fish are gaining use as alternative models for chemical carcinogenesis studies. Many contributing factors include short life span, short time to tumor response, ability to spawn regularly, reduced husbandry costs, the ability to include the whole fish sagittal-section on one microscope slide for histology, and a low spontaneous tumor incidence (Hawkins *et al.*, 1988). The zebrafish was the first aquarium species in which chemically-induced tumors were demonstrated (Stanton, 1965). Later studies confirmed their responsiveness to diethylnitrosamine and also to nitrosomorpholine (Khudoley, 1984; Pliss and Khudoley, 1975). However, subsequent investigations have focused on life stage effects and toxicity upon exposure to various chemicals and heavy metals (Braunbeck *et al.*, 1990a; 1990b; Nagel *et al.*, 1991, Kalsch *et al.*, 1991; Bresch *et al.*, 1990; Nielson *et al.*, 1990; Dave and Xiu, 1991), with surprisingly little work on the response of zebrafish to carcinogens.

Aflatoxins are secondary metabolites produced by the fungus *Aspergillus flavus*, with aflatoxin B₁ identified as the most toxic metabolite. AFB₁ is a potent hepatocarcinogen and hepatotoxin in some species, such as the rat, duck, and rainbow trout, while other species, such as the mouse, seem to be fairly resistant to its effects. AFB₁ has recently been classified as a Group 1 carcinogen in humans (IARC, 1993). Aflatoxin exposure may occur via contaminated food, such as moldy corn and peanuts. Research has indicated that interspecies differences in sensitivity to this hepatocarcinogen can often be explained by variations in the biotransformation of AFB₁ (Bechtel, 1989; Bailey *et al.*, 1996). The activation of AFB₁ to the 8,9-epoxide is thought to be responsible for its carcinogenic effects because this unstable, highly reactive intermediate can bind to cellular macromolecules (Essigman *et al.*, 1982; Swenson *et al.*, 1977). However, formation of this intermediate alone is not enough to account for carcinogenesis, since detoxification is possible via a glutathione-S-transferase mediated reaction. Successful scavenging of the intermediate by this pathway has been shown to be responsible for lower susceptibility to the carcinogenic effects of AFB₁ in mouse compared to rat (Degen and Neumann, 1981).

AFB₁ response in fish depends upon the species being investigated. For example, coho salmon and catfish are resistant but rainbow trout are one of the most sensitive species known to the carcinogenic effects of AFB₁ (reviewed in Hendricks, 1994; Bailey *et al.*, 1996). A study in 1968 showed that exposure to microgram quantities of AFB₁ were acutely toxic to zebrafish embryos and larvae (Abedi and McKinley, 1968). However, zebrafish did not develop tumors after 2 ppm water exposure for 3 days (Bauer *et al.*, 1972). We have recently found AFB₁ to be a relatively weak hepatocarcinogen in this species (unpublished results). The purpose of this study was to characterize the *in vivo* metabolism and hepatic DNA adduction of AFB₁ in the zebrafish as a first step in assessing the mechanisms of AFB₁ resistance in this species.

MATERIALS AND METHODS

Animals.

Adult male and female zebrafish (*Danio rerio*) were obtained from the Food Toxicology and Nutrition Laboratory at Oregon State University. The zebrafish were maintained in 29 gallon aerated aquariums and a controlled temperature of 26°C (± 1) and a 14 hour light:10 hour dark photoperiod. During the week, fish were fed twice daily with TetraMin Staple Food (flakes) each morning, and a combination of Oregon Test Diet (OTD) (Sinnhuber *et al.*, 1977) and brine shrimp in the afternoon on Monday, Wednesday, and Friday, and TetraMin Staple Food and brine shrimp on Tuesday and Thursday afternoons. On weekends, fish received only one feeding per day consisting of OTD and brine shrimp.

Fingerling rainbow trout (*Oncorhynchus mykiss*) were reared in the Food Toxicology and Nutrition Laboratory as described (Sinnhuber *et al.*, 1977).

Chemicals.

[³H(G)]Aflatoxin B₁ (AFB₁) was purchased from Moravsek Biochemicals (Brea, CA); Proteinase K, and RNase, DNase free were from Boehringer Mannheim Biochemicals (Indianapolis, IN); HPLC grade acetonitrile, methanol, tetrahydrofuran, and J.T. Baker C₁₈ Empore extraction disks were from VWR (Seattle, WA); Hoechst #33258 from Calbiochem-Behring Corp. (La Jolla, CA); aflatoxin HPLC standards aflatoxicol (AFL), aflatoxicol-glucuronide (AFL-g), aflatoxin-M₁ (AFM₁), and aflatoxicol-M₁ (AFL-M₁) were prepared in our laboratory by previously published methods (Loveland *et al.*, 1983; 1984); all other chemicals were purchased from Sigma (St. Louis, MO) or Aldrich Chemical Company (Milwaukee, WI).

Metabolism of [³H]AFB₁.

Adult female zebrafish, fasted for 24 hours, were intraperitoneally (i.p.) injected with 400 µg/kg body weight of [³H]AFB₁ (11.6 Ci/mmol) in 50% ethanol. The fish were rinsed with 2 ml of water after injection to account for leakage, and placed in the dark in individual beakers containing 50 ml water. Water samples were collected and counted at various time points up to 24 hours for metabolite analysis. AFB₁ and metabolites were extracted from the aqueous samples using C₁₈ Empore Extraction Disks. The disks were washed with 45 ml of the elution solvent (10 mM potassium acetate (KOAc), pH 5.0, adjusted to 60% methanol (MeOH)) in 15 ml increments, and then conditioned with 45 ml of 100% MeOH, 45 ml of 50% MeOH, and 45 ml of 10% MeOH. Water samples were adjusted to 10% methanol and were loaded onto the conditioned disks. Prior to elution, the disks were washed with 10 ml of 10 mM KOAc, pH 5.0, adjusted to 10% MeOH, to wash off proteins, salts, and exchanged tritium. AFB₁ and metabolites were eluted with 15 ml of 10 mM KOAc, pH 5.0, adjusted to 60% methanol. Total recovery rates (comprised of all fractions and the disk) were greater than 85%, and the amount of radioactivity associated with the 60% methanol eluant represented 60-88% of the total radioactivity. The methanol eluants, containing the aflatoxin metabolites, were concentrated using a rotovap and redissolved in 15% acetonitrile: methanol: tetrahydrofuran (AMT; 15:20:6 v:v:v) and 85% 0.02 M KOAc, pH 5.0. Reverse phase high pressure liquid chromatography was performed with a Beckman Model 334 instrument using a 4.6 x 150 mm, 5 micron Phenomenex C₁₈ column. The solvent system consisted of AMT (15:20:6 v:v:v) and 0.02 M KOAc, pH 5.0, with a 15 minute linear gradient from 15% AMT to 46% AMT. The flow rate was 1 ml/min, and UV detection was at 345 nm. Effluent samples of 30 drops (0.3 ml) were collected for liquid scintillation counting using a Beckman LS 7500 scintillation counter.

Hepatic aflatoxin-DNA adduction.

To evaluate the dose-response of AFB₁-DNA binding, adult male and female zebrafish fasted for 24 hours were i.p. injected with 0, 50, 100, 200, or 400 µg/kg body weight of [³H]AFB₁ (11.6 Ci/mmol) in 50% EtOH and were rinsed with 2 ml of water to account for leakage from the injection site. Livers were sampled 24 hours after injection and were immediately frozen in liquid nitrogen. DNA isolation and purification was based on the procedure by Strauss (1991), with the following modifications. Liver chunks were homogenized with digestion buffer. The samples were first extracted with equal volumes of phenol saturated with Tris-HCl buffer, pH 8.0., and then extracted twice with equal volumes of chloroform: isoamyl alcohol (24:1). The DNA was precipitated using 0.1 volume of 3 M sodium acetate and 2 volumes of cold 100% EtOH. After centrifugation, the EtOH was removed and the DNA allowed to dry. The DNA was dissolved in 0.5 ml of millipore water by heating the samples at 38°C for 30 minutes, and then RNase, DNase-free was added to the sample (final concentration 5µg/ml), and the incubation continued for another 30 minutes. The samples were extracted twice with equal volumes of chloroform: isoamyl alcohol (24:1), and the DNA was again precipitated. After centrifugation, the EtOH was removed, and the DNA was washed with 70% ice-cold EtOH. Samples were stored at -20°C after dissolving the DNA in TE buffer, pH 8.0. DNA concentration was quantified using the microfluorometric procedure described by Cesarone *et al.* (1979), using a 33258 Hoescht fluorometer. To determine the amount of [³H]AFB₁ bound to the DNA, samples were hydrolyzed by mixing with equal volumes of 1.0 M perchloric acid and heating at 70°C for 20 minutes. The hydrolyzed samples were then counted with a scintillation counter.

To verify the sex-related difference in DNA binding observed in the dose-response study, an experiment was conducted with 9 groups of 3 females, and 7 groups of 3 males. The fish were i.p. injected with 400 µg/kg body weight of [³H]AFB₁ (13.0 Ci/mmol), the highest dose used in the dose-

response experiment. Livers were sampled 24 hours later, and the amount of hepatic DNA binding was determined.

To evaluate the time-course of AFB₁-DNA binding, adult male and female zebrafish were i.p. injected with 400 µg/kg body weight of [³H]AFB₁ (15.4 Ci/mmol), and livers were sampled 1, 2, 4, 7, 14, and 21 days after injection. Livers were immediately frozen in liquid nitrogen. DNA binding was determined as described above.

In vitro AFB₁-metabolism assay.

The *in vitro* metabolism of AFB₁ was carried out with juvenile trout (n=10), or adult female (n=20) or male (n=6) zebrafish liver homogenates. The fish were fasted for 24 hours before sampling. Female zebrafish and juvenile trout livers were excised and immediately frozen in liquid nitrogen and stored at -80°C. Male zebrafish livers were immediately placed into homogenization buffer and homogenized and then frozen due to difficulties with protease release during liver excision. Livers were homogenized in a buffer containing 0.1 M potassium phosphate (pH 7.25), 20% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 0.1 mM butylated hydroxytoluene (BHT) on ice. Samples were centrifuged at 1,000x g for 10 minutes, and the supernatant decanted and stored at -80°C. Protein concentration was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

The *in vitro* AFB₁-metabolism assay was based on the method of Monroe and Eaton (1987), as modified by Takahashi *et al.* (1996). Samples were preincubated for 2 minutes at room temperature before initiating the reaction with NADPH and reduced glutathione. Incubations were carried out for 45 minutes. Michaelis-Menton kinetics were determined with trout homogenates at both 13°C and 28°C using substrate (AFB₁) concentrations of 5, 10, 20 and 40 µM AFB₁, and with female zebrafish homogenates at 28°C at substrate concentrations of 10, 20, 40 and 80 µM AFB₁. Male zebrafish

homogenates were assayed using 80 μM AFB₁, the highest substrate concentration, for comparison to female zebrafish. All assays were conducted in duplicate. Incubations without homogenates were used to correct for the low inherent epoxidation activity of butylated hydroxyanisole (BHA)-induced mouse cytosolic protein included in the assay mixture.

Statistical analysis was performed using SAS, version 6.8. Analysis was performed using either a one-way or two-way analysis of variance. A p value of less than 0.05 was considered significant.

RESULTS

In vivo metabolism of [³H]AFB₁.

Early excretion kinetics of [³H]AFB₁ were evaluated by measuring the amount of radioactivity recovered in water within a 24 hour time period after i.p. injection (Fig. 1). By 24 hours, 47% of the radioactivity administered to the zebrafish was recovered in the water. Excretion occurred rapidly with 25% of the administered radioactivity recovered by 45 minutes. This amount represented more than half of the total radioactivity recovered in the 24 hour time period. The remaining 53% of the administered dose is presumed to represent material found bound strongly to macromolecules, and material eventually eliminated via fecal excretion.

The major metabolites of AFB₁ recovered in the water at various time points after i.p. injection were identified as aflatoxicol (AFL), aflatoxicol-glucuronide (AFL-g), and unreacted parent AFB₁ (Fig. 2). Identification of these peaks was based on the retention times of known aflatoxin standards generated in our lab. Formation and excretion of AFL occurred rapidly in the zebrafish and was present in the water at 5 minutes, the earliest time point examined. Over the 24 hour time-period, AFL was the predominant metabolite, accounting for 17.3% of the original dose of AFB₁ administered. AFL-g and unreacted AFB₁ accounted for 4.1% and 3.6%, respectively.

Figure 3 shows the proportion of AFB₁ and metabolites recovered in the HPLC profile at each time point investigated, with values normalized to 100. AFL was the major metabolite recovered in the water at each time point up to 18 hours, accounting for 60 to 80% of the metabolites recovered. After 18 hours, AFL-g was the major metabolite, and represented over 80% of metabolites recovered at the 24 hour time point. An unidentified polar peak accounting for only a minor percentage of the metabolites was occasionally detected, being most prominent at 5 minutes. While this peak could represent a more polar metabolite, it did not co-elute with authentic AFB₁-glutathione conjugate, and was not reproducible in another experiment conducted investigating aflatoxin metabolism (unpublished results). Unresolved

aflatoxicol-M₁ (AFL-M₁) plus aflatoxin-M₁ (AFM₁) represented only a very minor fraction of the metabolites, comprising less than 2% of the metabolites recovered at any one time point.

In vivo AFB₁-DNA adduction in liver.

The *in vivo* dose-response for AFB₁-DNA adduct formation was linear from 50-400 $\mu\text{g AFB}_1/\text{kg}$ body weight (Fig. 4). Females had a higher level of DNA binding than the males at all doses tested. The covalent binding index (CBI), where $\text{CBI} = \mu\text{mol chemical bound/mol DNA/mmol chemical administered/kg body weight}$, was $32,700 \pm 21,800$ for males, and $56,000 \pm 15,500$ for females. Females had a DNA binding index which was significantly higher (1.7 fold higher) than males ($p < 0.01$). A follow-up study investigating sex-related difference in AFB₁-DNA adduction verified the results found in the dose-response experiment. The binding index in the second experiment was $68,000 \pm 31,400$ for females and $29,100 \pm 12,600$ for males, which represents a 2.3 fold higher binding index in females compared to males ($p < 0.05$).

An investigation of the time-course of AFB₁-DNA binding was conducted to assess when maximum DNA adduct formation occurred, and to investigate the persistence of DNA adducts in this species (Fig. 5). Although there was some trend toward highest adduction at day 7, the time-course experiment failed to show any statistical difference in binding levels from 1 to 21 days. Therefore, the time at which maximal adduct formation occurs could not be calculated. The half-life of the DNA adducts was also not evident from this experiment. However, a significant difference was again noted between male and female AFB₁-DNA binding levels, with the females exhibiting a binding index on average 42,000 units higher than the males ($p < 0.001$).

In vitro AFB₁-metabolism assay.

Trout liver homogenates exhibited a higher V_{max} and K_m for oxidation of AFB₁ to the 8,9-epoxide at both 13°C and 28°C compared to the female zebrafish. As was expected, trout V_{max} and K_m decreased at the lower temperature. However, the values at 13°C are probably more representative of the physiological response in the trout, since this is their normal environmental temperature. At 13°C, the trout liver homogenates had a 3.1 fold higher V_{max} and a similar K_m when compared to female zebrafish homogenates at 28°C (Table 1) in this experiment. The 7-fold lower K_m for AFL production suggests that the reaction would predominate over AFB₁-8-9-epoxide formation at low AFB₁ concentrations *in vivo* in zebrafish, and is consistent with the rapid excretion of AFL within 5 minutes of AFB₁ treatment.

Not surprising was the relatively high amount of aflatoxicol which was formed in the zebrafish liver homogenates. At 80 µM AFB₁, male and female liver homogenates exhibited comparable activities for aflatoxicol production, with the female zebrafish liver homogenates possessing an activity of 8.17 ± 0.09 pmol/min/mg protein, and male zebrafish an activity of 8.70 ± 0.04 pmol/min/mg protein. Male zebrafish did appear to have a lower activity towards formation of the AFB₁-epoxide than females (4.10 ± 0.08 pmol/min/mg protein compared to 5.90 ± 0.10 pmol/min/mg protein, $p < 0.01$).

DISCUSSION

The results demonstrate that zebrafish have the capacity for both Phase I and Phase II metabolism of AFB₁. The major *in vivo* metabolites excreted into water were aflatoxicol and aflatoxicol-glucuronide, followed by unreacted AFB₁. This metabolic profile is similar to that seen in other fish species, such as the Japanese medaka, rainbow trout, and channel catfish (Toledo *et al.*, 1987; Gallagher and Eaton, 1995; Loveland *et al.*, 1984). Aflatoxicol, produced by a cytosolic reductase reaction, was recovered in the water at 5 minutes, the earliest time point examined. This has also been observed in the Japanese medaka (Toledo *et al.*, 1987). It is important to note that while AFL is a polar metabolite of AFB₁, it has been shown to be 70% as mutagenic and just as carcinogenic as AFB₁ in rainbow trout, and is also a potent carcinogen in Fischer 344 rats (Coulombe *et al.*, 1982; Schoenhard *et al.*, 1981; Nixon *et al.*, 1981; Bailey *et al.*, 1994). The major conjugation (Phase II) reaction was glucuronidation, which is an important reaction in eliminating hydroxylated compounds in fish (Clark *et al.*, 1991). No evidence of glutathione conjugation was present, as shown by co-chromatography with a glutathione conjugate standard. Formation and excretion of the aflatoxin metabolites occurred rapidly in the zebrafish after i.p. injection.

The *in vitro* assay assessing AFB₁ metabolism again demonstrated the proficiency of aflatoxicol formation in zebrafish. This assay also evaluated the ability for AFB₁ epoxidation to the reactive intermediate by trapping the epoxide as the aflatoxin-glutathione adduct formed. The results from this assay revealed that zebrafish possess the enzymes necessary for bioactivation of this carcinogen. When compared to trout liver homogenates assayed at 13°C, zebrafish appear to have a comparable K_m and only a 3-fold lower V_{max}, which suggests that zebrafish should be a fairly sensitive species for AFB₁ carcinogenesis. Buchmann *et al.* (1993) conducted immunohistochemical analysis to detect the presence of the cytochrome P450 isozyme LMC2 (CYP2K1) in zebrafish. This isozyme is believed to be primarily responsible for the bioactivation of AFB₁ to the epoxide in rainbow trout (Williams and Buhler, 1983).

The immunohistochemical analysis of zebrafish using antibodies directed against trout CYP2K1 showed that this isozyme was constitutively expressed in the liver, kidney, skin, and oral mucosa of zebrafish, with lower expression in gills, pseudobranch, intestines, and ovaries. It is possible that this or a similar P450 may be responsible for the observed bioactivation of AFB₁ in zebrafish.

A significant difference in DNA adduct formation between male and female zebrafish was observed in all DNA binding experiments performed. An *in vitro* AFB₁ metabolism assay also confirmed the difference between males and females in their ability to bioactivate AFB₁. Differences between sexes in their ability to metabolize xenobiotics are not unusual. A difference in xenobiotic biotransformation between male and female zebrafish was previously noted by Buchmann *et al.* (1993), where males tended to have higher 7-ethoxyresorufin-*O*-deethylase activity. This activity is usually associated with the presence of cytochrome P4501A. The *in vivo* dose-response of hepatic aflatoxin-DNA adduct formation was linear from 50-400 µg AFB₁/kg body weight, which is consistent with experiments examining hepatic DNA binding in other fish species following AFB₁ administration (Dashwood *et al.*, 1988; Toledo *et al.*, 1987; and Witham *et al.*, 1982). The time-course of aflatoxin-DNA binding using the highest dose showed no statistical difference in binding levels from 1 to 21 days, suggesting slow DNA repair in zebrafish liver. A useful method for comparing DNA binding between species is the CBI (cumulative binding index, units of µmol chemical bound/mol DNA/mmol chemical administered/kg body weight). A composite CBI calculated from the three DNA-binding experiments is approximately 70,600 in female zebrafish and 35,000 for males. For comparison, the CBI for rat and Japanese medaka is approximately 10,000 and 13,000, respectively, and is 240,000 for trout (Toledo *et al.*, 1987, and references therein). From these data, zebrafish exhibit approximately a 4-fold lower capacity for DNA adduct formation compared to trout, and a 5-fold higher capacity than rat and medaka.

The results from both the investigation of *in vivo* DNA binding after i.p. administration of AFB₁ and the *in vitro* AFB₁ metabolism assay suggest that zebrafish should be a fairly sensitive model for AFB₁

carcinogenesis, if indeed the amount of DNA adducts present in this species truly correlates with tumor incidence as demonstrated in the trout model (Dashwood, *et al.*, 1992). Not only are zebrafish capable of bioactivating AFB₁ to its active epoxide form, but the DNA adducts that result seem to be persistent over time. However, a study conducted in our laboratory investigating dietary exposure to AFB₁ is providing evidence to the contrary. Zebrafish appear to be quite resistant to the carcinogenic effects of AFB₁ when administered by the dietary route (unpublished results). A preliminary investigation of DNA binding after dietary administration of [³H]AFB₁ showed only a low level of adduct formation (unpublished results). This dramatic difference is suggestive of a difference in absorption. It appears that if one could get the carcinogen to the target organ, zebrafish might prove quite sensitive. Studies assessing carcinogenicity of i.p. administered AFB₁ have yet to be conducted. It may also be that, while there is evidence for persistence of DNA adduct formation after i.p. administration, these adducts simply do not go on to form initiated cells. Finally, even if initiation occurs, there could be a lack of promotion/progression of these cells in zebrafish liver. Further studies will be required to test these hypotheses.

Other studies conducted in our laboratory with dietary administration of high amounts of diethylnitrosamine, dimethylnitrosamine, or dimethylbenz[*a*]anthracene for 6 months have shown limited or no tumor response as well (unpublished results). However, high doses of dietary methylazoxymethanol acetate did manage to elicit almost a 30% tumor response (unpublished results). Detailed metabolism studies have not been conducted after administration of these various carcinogens, so it is uncertain if these low tumor responses are the product of low capacity for bioactivation, high capacity for detoxification and elimination, inability of these compounds to reach the respective target organs, or some other factor governing the carcinogenic process. Interestingly, zebrafish exposed as embryos or fry to many of these carcinogens are proving to be more sensitive, indicating that zebrafish may be more susceptible to carcinogens at an early stage of development.

In conclusion, adult zebrafish can rapidly metabolize and excrete the potent hepatocarcinogen AFB₁ after intraperitoneal administration. Both *in vitro* and *in vivo* studies verify that this species has the capacity to bioactivate AFB₁ to its reactive intermediate. The DNA adducts that result are suggestive of sensitivity to this carcinogen. These results are in apparent contradiction to the resistance of zebrafish to dietary administration of AFB₁, and suggest a mechanism related to factors other than inherent ability to metabolize and bioactivate this carcinogen.

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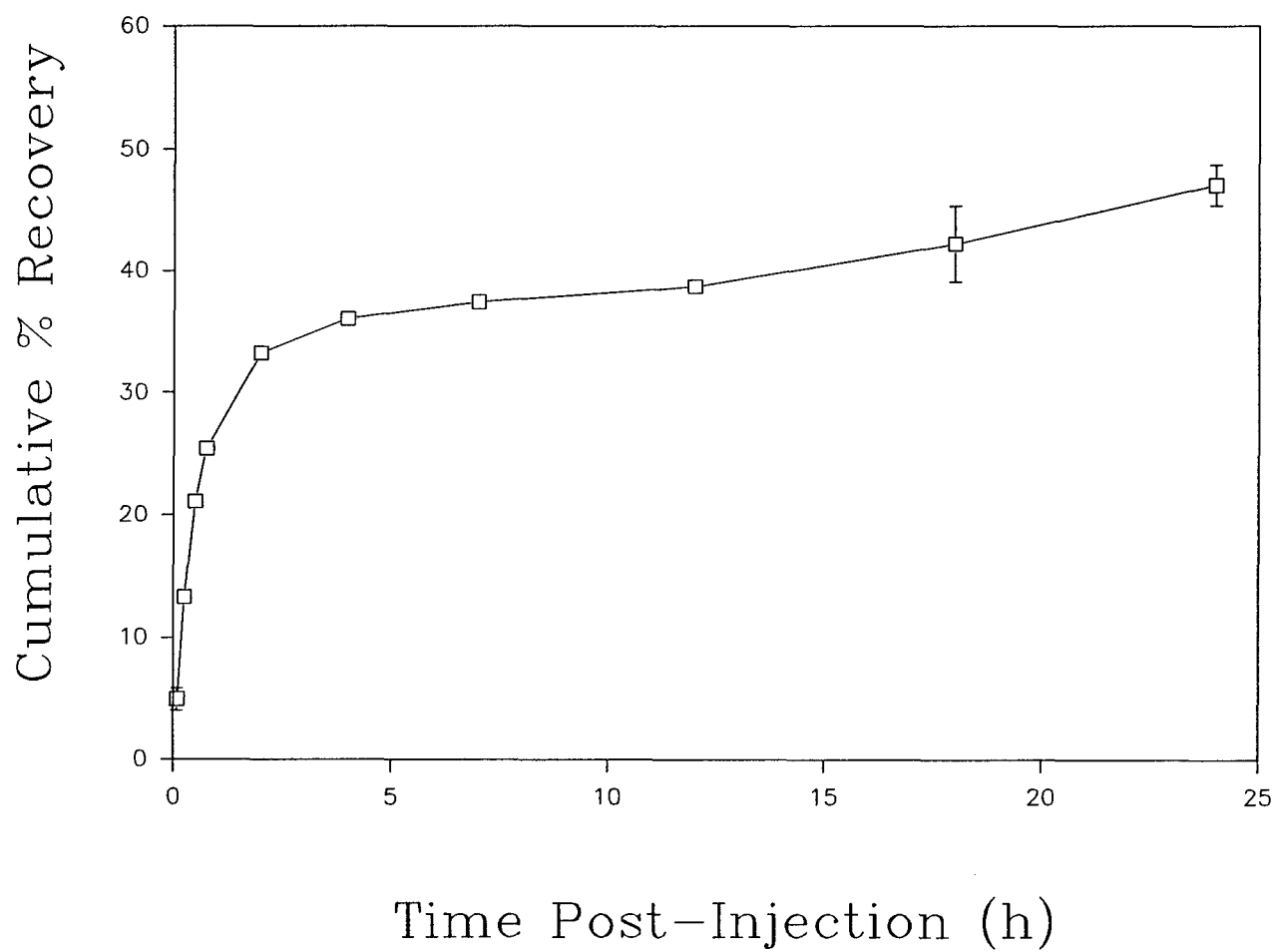


Fig. 1. The cumulative percentage of radioactivity excreted into water within 24 hours after i.p. injection of 400 μg AFB₁/kg body weight (n=3 females, error bars are \pm SEM).

Figure 2

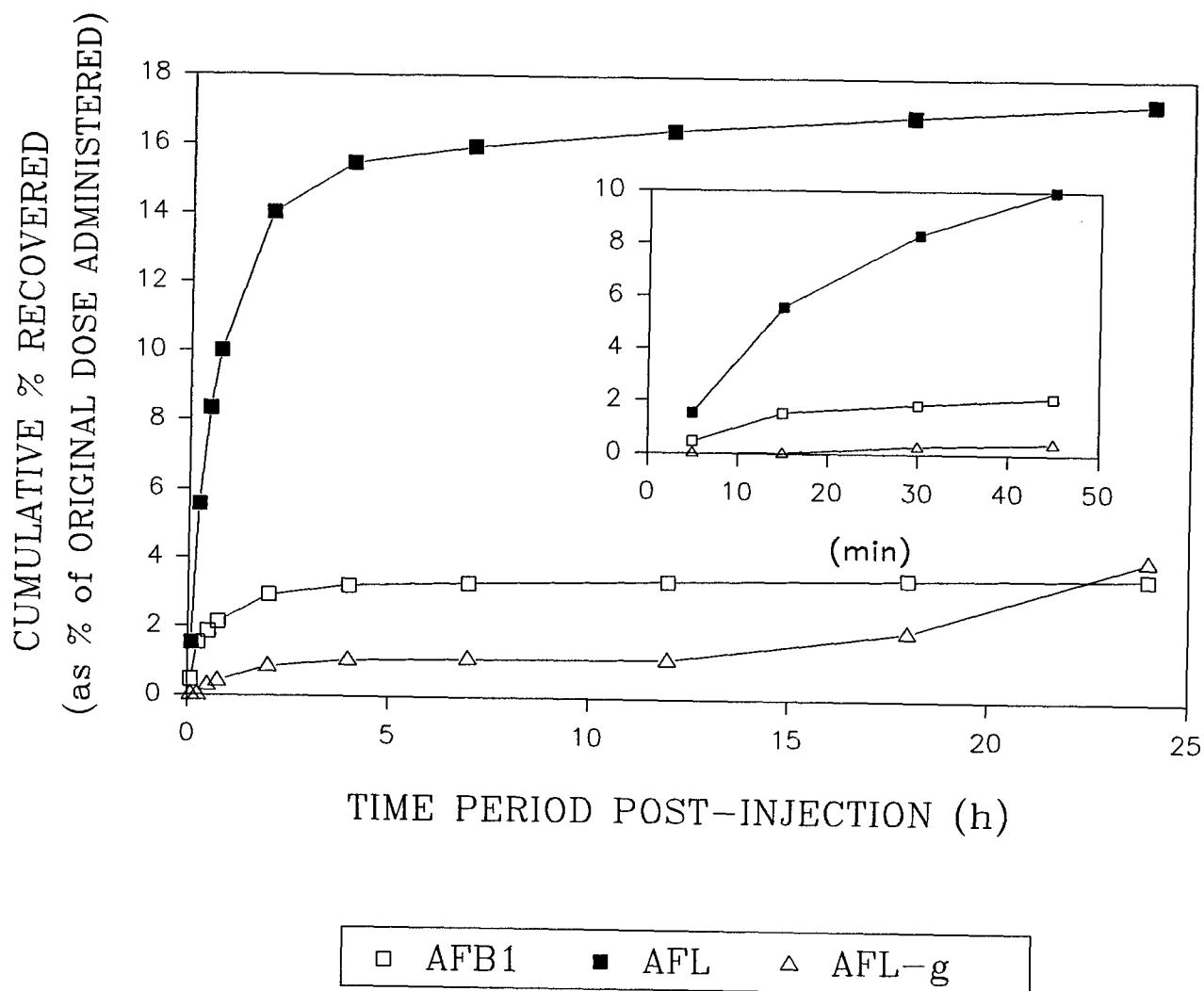


Fig. 2. The cumulative percentage of AFB₁ and metabolites excreted into water as determined by HPLC as a percentage of the original dose (400 μ g [³H]AFB₁/kg body weight) administered. Each data point represents the pooled water samples from 3 females.

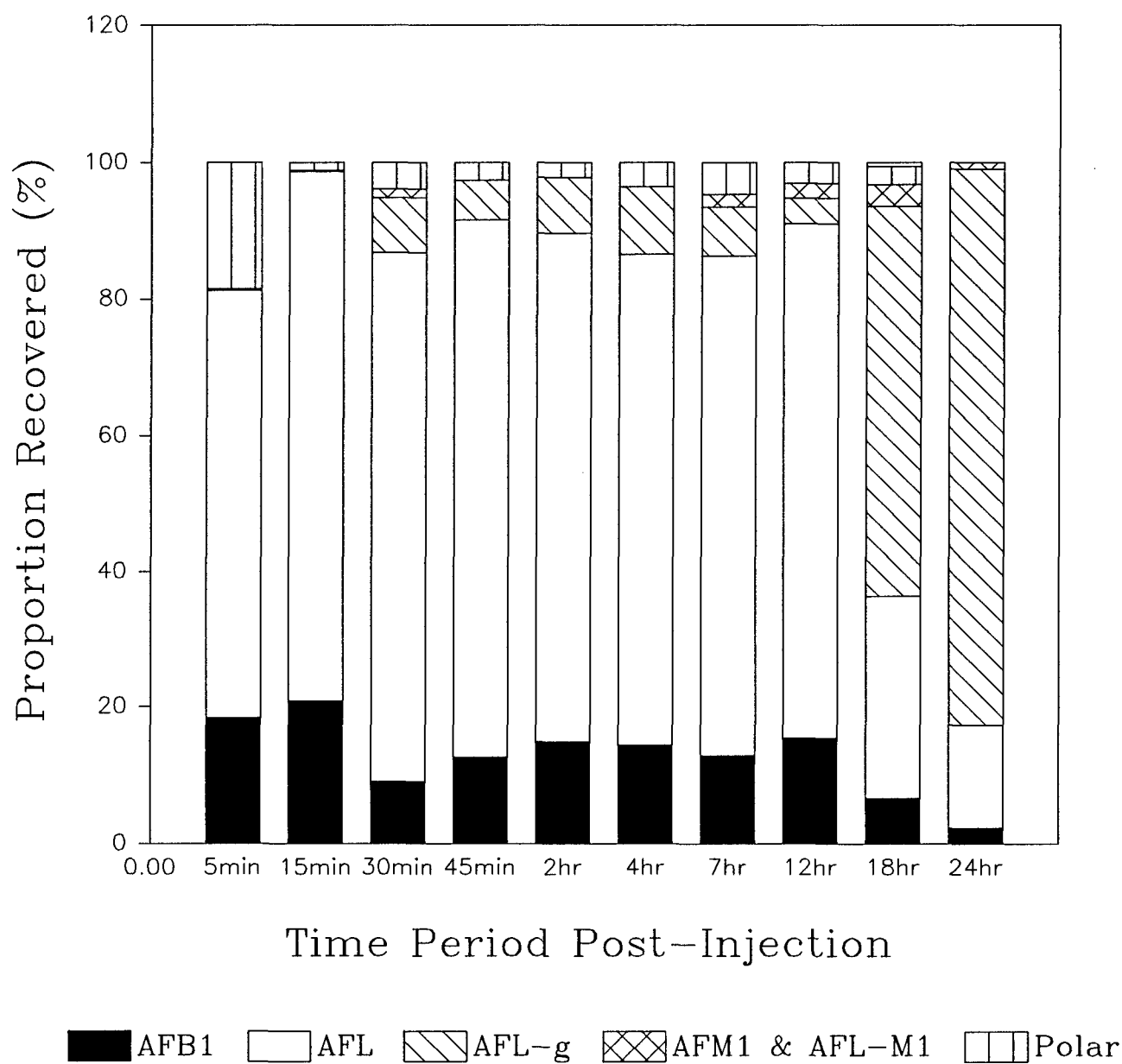


Fig. 3. The proportion of AFB₁ and metabolites excreted into water at various time points over 24 hours as determined by HPLC. Values are normalized to 100.

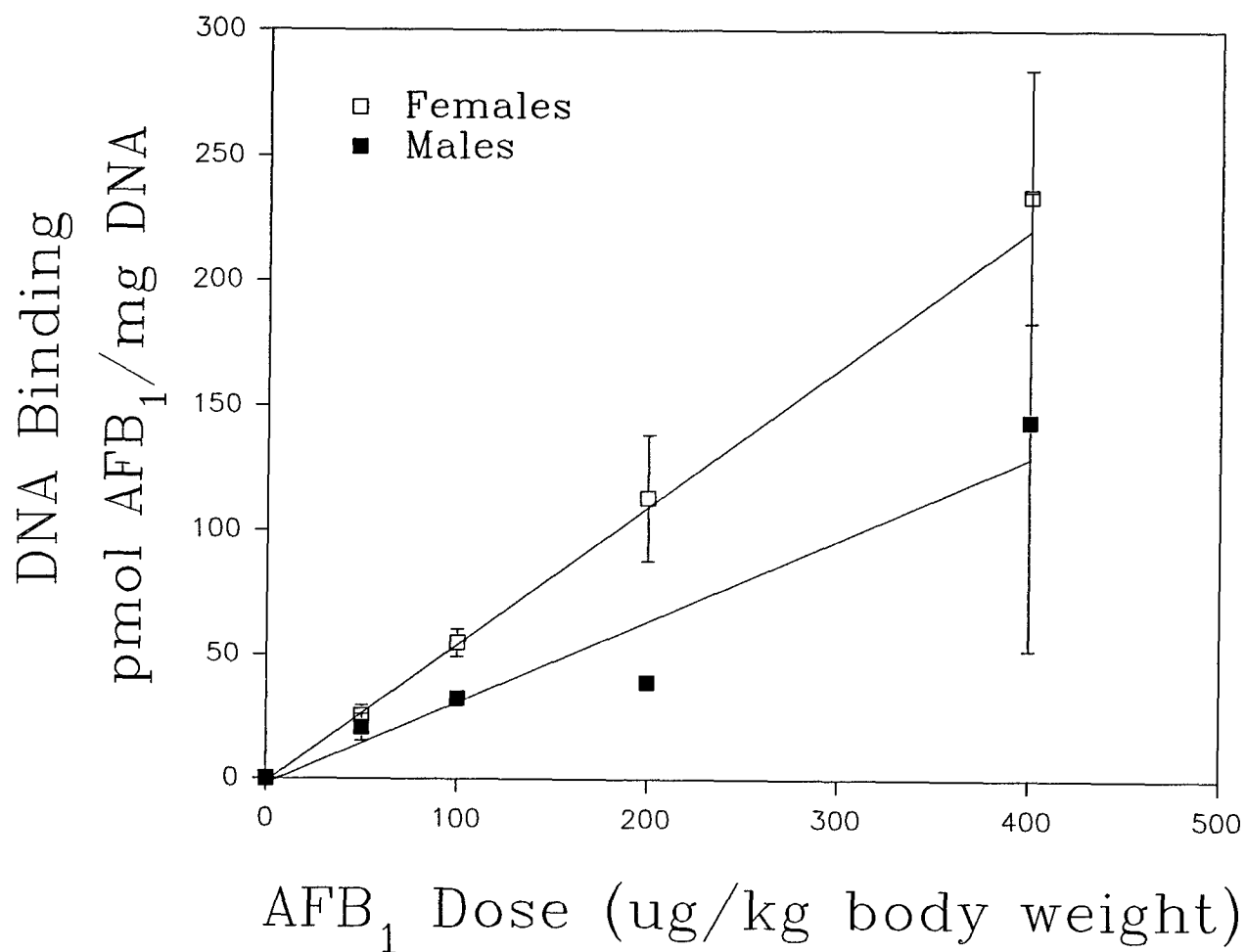


Fig. 4. The *in vivo* dose response of hepatic aflatoxin-DNA adduct formation at 24 hours. Fish were i.p. injected with 50-400 μg [^3H]AFB₁/kg body weight (data are means \pm SEM from 3 pools of 3 fish each.)

Figure 5

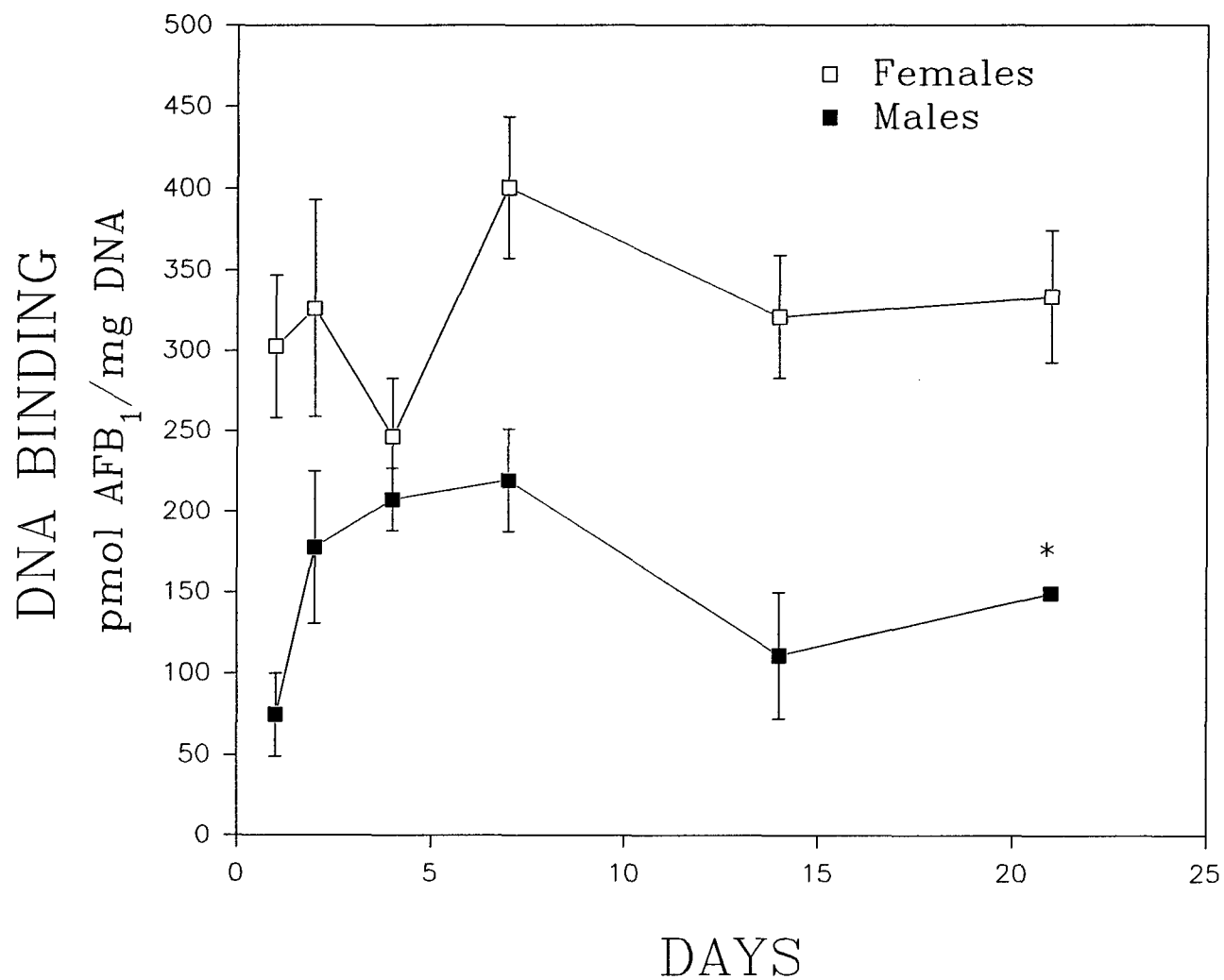


Fig. 5. Aflatoxin-DNA adduct formation from 1-21 days (data are means \pm SEM from 3 pools of 3 fish each; * denotes mean \pm SEM from 2 pools of 3 fish each).

Table 1. Michaelis-Menton kinetics of aflatoxin B₁-epoxide formation in rainbow trout and zebrafish liver homogenates and aflatoxicol formation in zebrafish liver homogenates with the substrate AFB₁^a.

Species	Temperature	K _m (μ M)	V _{max} (pmol/min/mg)
Production of AFB ₁ -8-9-epoxide:			
Rainbow trout	13°C	81.2 \pm 12.8	35.9 \pm 4.1
Rainbow trout	28°C	109.3 \pm 15.0	66.6 \pm 7.1
Zebrafish (female)	28°C	79.0 \pm 16.4	11.7 \pm 1.4
Production of AFL:			
Zebrafish (female)	28°C	11.2 \pm 1.3	9.1 \pm 0.3

^a n=10 for trout, and n=20 for zebrafish; all assays were run in duplicate, with nonenzymatic background activity subtracted; errors are given as asymptotic standard error. Only female zebrafish were used for this comparison against juvenile rainbow trout, which do not show sexual differences in AFB₁ metabolism prior to maturity.

Cytochrome P4501A Induction by β -Naphthoflavone, Aroclor 1254, and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, and Its Influence on Aflatoxin B₁ Metabolism and DNA Binding in Zebrafish (*Danio rerio*).

Claudia M. Troxel¹, Donald R. Buhler,^{1,2,4} Jerry D. Hendricks^{1,3,4}, and George S. Bailey^{1,3,4,5}.

¹ Toxicology Program, Oregon State University

² Department of Agricultural Chemistry, Oregon State University

³ Department of Food Science and Technology, Oregon State University

⁴ Marine/Freshwater Biomedical Sciences Center

⁵ To whom correspondence should be addressed at Department of Food Science and Technology, Marine/Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, OR 97331.
Telephone: (541) 737-3164. Fax: (541) 737-1877. E-mail: baileyg@bcc.orst.edu

Cytochrome P4501A Induction by β -naphthoflavone, Aroclor 1254, and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, and Its Influence On Aflatoxin B₁ Metabolism and DNA Binding in Zebrafish (*Danio rerio*).

Troxel, C.M., Buhler, D.R., Hendricks, J.D., and Bailey, G.S. *Toxicol. Appl. Pharmacol.*

This study investigated the response of CYP1A in the zebrafish (*Danio rerio*) following exposure to Aroclor 1254, β -naphthoflavone (β NF), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and then investigated TCDD modulation of aflatoxin B₁ (AFB₁) metabolism and hepatic AFB₁-DNA adduction. Aroclor 1254 fed at 500 ppm for 1 to 9 days or intraperitoneal (i.p.) injection of 75-200 mg Aroclor 1254/kg body weight failed to induce CYP1A protein or associated 7-ethoxyresorufin-*O*-deethylase (EROD) activity. Dietary β NF at 500 ppm for 3 or 7 days induced CYP1A protein and EROD activity approximately 3-fold above controls. A single i.p. injection of 150 mg β NF showed maximal induction of CYP1A protein and EROD activity by 24 hours, both of which decreased rapidly during the next 6 days. A more refined study showed maximum CYP1A protein levels between 24 and 36 hours, while EROD activity had an observable peak at 16 hours. CYP1A and EROD activity showed dose-responsiveness following single i.p. administration of 25, 50, 100, or 150 mg β NF/kg body weight. Dietary exposure to 0.75 ppm TCDD for 3 days also significantly induced CYP1A. The effect of TCDD on the metabolism of AFB₁ in zebrafish was then investigated. The major AFB₁ metabolites excreted in water over 24 hours in the control group were aflatoxicol, aflatoxicol-glucuronide, and parent AFB₁. By contrast, the predominant metabolites in the TCDD-pretreated group were aflatoxicol-M₁-glucuronide, aflatoxicol, aflatoxin M₁ plus aflatoxicol-M₁ unresolved, aflatoxicol-glucuronide, and parent AFB₁. Surprisingly, hepatic AFB₁-DNA adduction was approximately 4-fold higher in the TCDD treated group compared to controls. This significant difference could not be explained by increased capacity for bioactivation of AFB₁ as measured by an *in vitro* AFB₁ metabolism assay. However, it was demonstrated that zebrafish have the capacity to bioactivate aflatoxin M₁ to a reactive intermediate, and it is proposed

that secondary bioactivation of this genotoxic intermediate may be responsible for the increased DNA binding.

Introduction

Fish are becoming important models in many areas in science, including developmental biology, genetics, neurobiology, aquatic toxicology, and carcinogenesis (Powers, 1989). The trout model has been and continues to be extensively characterized as an alternative nonmammalian vertebrate model in carcinogenesis research (for review see Bailey *et al.*, 1996). The use of smaller fish as experimental models is also assuming prominence because they have several advantages over the trout model such as their small size, ability to spawn regularly, reduced husbandry costs, and their overall hardiness (Hawkins *et al.*, 1988). Many small fish species, such as the guppy, Japanese medaka, and zebrafish are proving to be susceptible to numerous carcinogens, including diethyl- and dimethylnitrosamine, 2-acetylaminofluorene, nitrosomorphiline, and methylazoxymethanol acetate (Sato *et al.*, 1973; Khudoley, 1984; Fournie *et al.*, 1987; Nakazawa *et al.*, 1985; Hawkins *et al.*, 1986; Stanton, 1965). Zebrafish are a particularly attractive fish model because they are also being extensively developed in both genetics and developmental biology. Studies in our laboratory investigating the sensitivity of zebrafish to carcinogens are demonstrating that this species is a complex model. Adult zebrafish appear to be somewhat resistant to carcinogens administered in the diet, but zebrafish are susceptible to carcinogen exposures in the water, particularly when exposed as embryos and fry (unpublished results). Induction of cytochrome P4501A (CYP1A) in fish is being investigated as a biomarker of exposure to certain compounds commonly found in the environment such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and halogenated dibenzo-*p*-dioxins and dibenzofurans (for review see Goksoyr, 1995). While fish appear to lack the equivalent of inducible phenobarbital-type P450s, most fish species do possess a CYP1A-like P450 that can be effectively induced to a high level (Goksoyr *et al.*, 1991; Stegeman, 1989; Stegeman and Hahn, 1994). CYP1A is not normally constitutively expressed in significant levels. Following induction, it is primarily located in hepatic tissue, although in fish, it can also be found in extrahepatic tissues such as gill, kidneys, gut and heart (Buchmann *et al.*, 1993; Stegeman *et al.*, 1989; Husoy *et al.*,

1994). Induction of CYP1A occurs following exposure to a wide range of compounds, including the classic inducer 3-methylcholanthrene, many flavones and indoles, and the PAHs and PCBs. In mammals, the Ah-receptor is the mediator of induction, and is known to be responsible not only for the induction of CYP1A1, but a host of other enzymes including quinone-oxido-reductase, glucuronosyltransferase, glutathione-S-transferase, CYP1A2, and CYP2B1 (Poland, 1979; Hankinson, 1995).

The enhanced metabolic capabilities following exposure to one of the many CYP1A-inducing agents may result in altered metabolism of other xenobiotics including procarcinogens. One of the most extensively characterized procarcinogens is the potent mycotoxin aflatoxin B₁ (AFB₁), which requires bioactivation to the reactive intermediate, the AFB₁-8-9-epoxide. This mycotoxin is carcinogenic in many species, including rat, human, and rainbow trout, the most sensitive species. Numerous investigations have explored the modulation of aflatoxin B₁ metabolism and carcinogenesis by compounds known to act via the Ah receptor. Studies with rainbow trout have shown that preexposure to the synthetic flavone β -naphthoflavone (β NF), the PCB mixture Aroclor 1254, and the natural plant constituent indole-3-carbinol reduces AFB₁ carcinogenesis by altering the metabolism of AFB₁ and reducing DNA adduction (Shelton *et al.*, 1986; Goeger *et al.*, 1986; Nixon *et al.*, 1984; Takahashi *et al.*, 1995; 1996). An *in vitro* investigation of β NF-induced channel catfish microsomes showed induction of CYP1A enhanced detoxification of the carcinogen, but did not affect bioactivation of AFB₁ to its reactive intermediate (Gallagher and Eaton, 1995). Other studies investigating β NF-induced rabbit pulmonary and hepatic microsomes and β NF-induced guinea pig liver, kidney, and lung microsomes have also shown increased formation of the less toxic metabolite aflatoxin M₁ and reduced DNA adduction (Daniels and Massey, 1992; Liu, *et al.*, 1993). Although 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent Ah receptor agonist known, surprisingly few studies have investigated the modulation of AFB₁ metabolism and DNA adduction by pre-exposure to TCDD. The purpose of this study was to characterize CYP1A induction in zebrafish by Aroclor 1254, β NF, and TCDD. After characterizing CYP1A induction,

further studies were conducted to investigate the *in vivo* modulation of AFB₁ metabolism and hepatic DNA adduction following dietary exposure to TCDD at a concentration known to exert biological effects on metabolizing enzymes.

Materials and Methods

Chemicals

[³H(G)]Aflatoxin B₁ (AFB₁) was purchased from Moravek Biochemicals (Brea, CA); βNF from Fluka Chemical Corp. (Ronkonkoma, NY); Aroclor 1254 from Monsanto Company (St. Louis, MO); TCDD from ANALABS (New Haven, CT); 7-ethoxyresorufin and resorufin from Molecular Probes, Inc. (Eugene, OR); RNase, DNase free from Boehringer Mannheim Biochemicals (Indianapolis, IN); HPLC grade acetonitrile, methanol, tetrahydrofuran, and J.T. Baker C₁₈ Empore extraction disks from VWR (Seattle, WA); Hoechst #33258 from Calbiochem-Behring Corp. (La Jolla, CA); aflatoxin HPLC standards aflatoxicol (AFL), aflatoxicol-glucuronide (AFL-g), aflatoxin M₁ (AFM₁), and aflatoxicol-M₁ (AFL-M₁) were prepared in our laboratory by previously published methods (Loveland *et al.*, 1983; 1984).

Animal care and maintenance

Adult (sexually mature) zebrafish were reared and maintained in the Food Toxicology and Nutrition Laboratory in aerated 29 gallon aquariums with a controlled temperature of 26°C (± 1) and a 14 hour light:10 hour dark photoperiod. During the week, fish were fed twice daily with TetraMin Staple Food (flakes) each morning, and a combination of Oregon Test Diet (OTD) (Sinnhuber *et al.*, 1977) and brine shrimp in the afternoon on Monday, Wednesday, and Friday, and TetraMin Staple Food and brine shrimp on Tuesday and Thursday afternoons. On weekends, fish received only one feeding per day consisting of OTD and brine shrimp. For dietary exposures, fish were fed one feeding of OTD or the test reagent in OTD per day during the course of the treatment. For the Aroclor 1254 dietary exposure only, fish were fed a control diet consisting of the purified casein diet (PC diet) or control diet containing Aroclor 1254 (DeKoven *et al.*, 1992).

Preparation of liver tissue

All fish were fasted for 24 hours before receiving the appropriate treatment. When fish were sampled, livers were immediately excised from the zebrafish and placed on ice until the appropriately sized pools of tissue were obtained. The samples were then flash frozen in liquid nitrogen and stored at -80°C. Before use, livers were homogenized in ice-cold buffer containing 0.1 M potassium phosphate (pH 7.25), 20% glycerol, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 0.1 mM butylated hydroxytoluene, and 0.1 mM phenylmethylsulfonylfluoride. The samples were centrifuged for 10 minutes at 1,000x g and the supernatant decanted for protein work. When DNA binding was also being investigated, the pellet was saved for DNA isolation and purification.

Investigation of CYP1A induction by Aroclor 1254, β NF, and TCDD

A total of 18 male and 18 female adult zebrafish were fed 500 ppm Aroclor 1254 twice per day and 3 males and 3 females each were sampled on 1, 2, 3, 5, 7, or 9 days. Four males and 4 females were fed the control PC diet twice per day and 1 male and 1 female sampled at days 2, 3, 5 and 7. When dietary exposure failed to show induction of CYP1A, 6 adult, female fish were i.p. injected with 100 or 200 mg Aroclor 1254/kg body weight, 4 were injected with carrier dimethylsulfoxide (DMSO), and all were sampled 24 hours later.

To confirm results from a preliminary investigation which showed CYP1A induction after i.p. exposure to β NF, a time-course study was undertaken. Thirty adult female zebrafish were i.p. injected with 150 mg β NF/kg body weight and sampled at day 1, 2, 3, 4, and 7; 6 controls were injected with DMSO and sampled at day 1, 2, and 3. A concurrent dietary exposure to 500 ppm of β NF was also conducted to investigate whether dietary exposure to this flavone was capable of inducing CYP1A in zebrafish. Sixteen adult female fish were fed 500 ppm β NF and 8 fish fed control diet for 3 or 7 days. To further pinpoint the time of maximum CYP1A induction by β NF, 32 adult female zebrafish were i.p.

injected with 150 mg/kg body weight of β NF in DMSO and sampled at 8, 16, 24, or 36 hours after injection. Twenty-four female zebrafish were also injected with 25, 50, or 100 mg/kg body weight of β NF in DMSO and sampled 24 hours later to try to establish a dose response (the 24 hour group in the time-course served as the 150 mg β NF/kg body weight dose). To evaluate if the use of DMSO instead of corn oil as a vehicle influenced the kinetics of induction, 40 females were i.p. injected with 150 mg/kg body weight of β NF in corn oil and sampled 24, 36, 48, 72, and 96 hours after injection. Lastly, to verify previous results demonstrating a lack of detectable CYP1A following exposure to Aroclor 1254, 16 female fish were i.p. injected with 75 or 150 mg Aroclor 1254/kg body weight using DMSO as a carrier. Ten females were i.p. injected with DMSO and 8 females were injected with corn oil and sampled 24 hours later to serve as controls for these experiments.

Finally, to verify that TCDD induces CYP1A in the zebrafish, 16 adult female zebrafish were fed 0.75 ppm TCDD or control diet for 3 days and sampled on the fourth day. This dosage was based on a previous study by Buchmann *et al.* (1993) which also investigated CYP1A in zebrafish.

TCDD modulation of aflatoxin DNA-binding

Sixty-four adult female zebrafish were fasted for 24 hours and then fed control OTD or OTD diet containing 0.75 ppm TCDD for 3 days. The day after feeding was completed, 8 of the control fish and 8 of the TCDD-treated fish were sampled for representative assessment of CYP1A levels and EROD activity at the time of AFB₁ dosage. Then, 12 of the control and 12 of the TCDD-treated fish were i.p. injected with DMSO and the other 12 control and 12 TCDD-treated fish were i.p. injected 400 μ g/kg body weight of [³H]AFB₁ (16.6 Ci/mmol) in DMSO. Fish were rinsed with 1 ml of water to account for skin contamination before placement in respective 5 gallon buckets containing approximately 16 liters of

aerated water. Rinse water accounted for less than 4% of the injected dosage. Livers were sampled 24 hours after injection.

DNA was isolated and purified using a modification of Strauss (1991) (modification found in Troxel *et al.*, publication in progress). DNA was quantified using the microfluorometric procedure of Cesarone *et al.* (1979) using a 33258 Hoescht DNA fluorometer. The amount of [^3H]AFB₁ bound to DNA was determined after hydrolyzing the DNA by heating the samples at 70°C with equal volumes of 1 M perchloric acid for 20 minutes and then counting with a Beckman LS 7500 scintillation counter.

TCDD modulation of AFB₁ metabolism

Twelve adult female zebrafish were fasted for 24 hours and then fed control OTD or OTD diet containing 0.75 ppm TCDD for 3 days. The day after feeding was completed, 3 control and 3 TCDD-treated fish were i.p. injected with DMSO, whereas the remaining 3 control and 3 TCDD-treated fish were i.p. injected with 400 µg/kg body weight of [^3H]AFB₁ (16.6 Ci/mmol) in DMSO. Fish were rinsed with 1 ml of water to remove any residual radioactivity before placement in individual beakers containing 50 ml of water. Rinse water contained less than 5% of the administered dose except for fish 2 of the AFB₁/TCDD treatment group, which contained approximately 8% of the injected dosage. At various time points, fish were removed to fresh beakers of water, the previous water samples were collected, and an aliquot was taken for scintillation counting. At 24 hours, the livers of the fish were sampled and treated as described earlier for enzyme analysis and quantification of DNA adduction.

Water samples were extracted and metabolite analysis performed by HPLC as described (Troxel *et al.*, publication in progress). The amount of radioactivity remaining after filtration by the C₁₈ (i.e., the amount not trapped by the filter) was higher than expected in some samples, so these water samples were refiltered with another C₁₈ filter. Some additional radioactivity was trapped in the 7 and 24 hour samples of the TCDD treated group, suggesting incomplete adsorption in the first filtration. However,

there still was a high amount of radioactivity remaining unbound in these doubly filtered water samples. To investigate the nature of this material, the 7 and 24 hour water samples from both groups were treated with 0.2 M sodium acetate buffer (pH 5.0), or buffer containing β -glucuronidase (4000 units/ml), or sulfatase (20 units/ml, with 40 mM D-saccharic acid-1,4-lactone added to inhibit β -glucuronidase activity) (Fong *et al.*, 1993). The samples were incubated at 37°C for 18 hours before extracting twice with 2 volumes of ethyl acetate. When analysis of the results showed some glucuronidation in the 7 and 24 hour sample from the AFB₁/TCDD treated group and the 24 hour sample from the AFB₁ group, up to 50 ml of the water samples were evaporated down to 1 ml, and the samples were then injected into the HPLC for further analysis. Under these HPLC conditions, AFM₁ and AFL-M₁ were not fully resolved, and therefore are reported together in the analysis.

Immunoblotting

Total protein was quantified using the method of Lowry *et al.* (1951) employing bovine serum albumin as the standard. Proteins were separated by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Due to the large number of samples to compare, after verification that only one cross-reacting band was present in blots analyzed for CYP1A protein, subsequent analysis of CYP1A was limited to dot blots. The blots were incubated with rabbit anti-trout IgG against LM_{4b} (CYP1A) at a concentration of 2 μ g/ml. The membranes were next probed with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. The P450 isozyme was detected using the ECL chemiluminescence detection kit. Densitometry analysis was performed with an HP ScanJet flatbed scanner using NIH Image version 1.54 software (Wayne Rasband, NIH, public domain). Previously quantified β NF-induced trout liver microsomes were used as standards on the blots, and all blots were normalized with the same sample of β NF-induced zebrafish liver homogenate.

Enzyme assays

EROD activity was determined by the method of Prough *et al.* (1978). The assay was conducted at 30°C using a substrate concentration of 2 μ M.

The *in vitro* AFB₁ metabolism assay was conducted using the method of Monroe and Eaton (1987), with modifications by Takahashi *et al.* (1996). This assay can indirectly measure bioactivation of AFB₁ by trapping the reactive intermediate with glutathione. Previous studies in which trout microsomes were incubated with AFM₁ instead of AFB₁ have shown the formation of another glutathione adduct with a different HPLC retention time than that of the AFB₁-glutathione adduct, suggesting that trout also bioactivate AFM₁ to a reactive intermediate (data not shown). In this experiment, the metabolism of both AFB₁ and AFM₁ was investigated in zebrafish liver homogenates. The final assay concentration of AFM₁ or AFB₁ was 80 μ M, and 0.51 mg/ml of supernatant protein from liver homogenates was used. Assay conditions consisted of a 2 minute pre-incubation at room temperature before the addition of the glutathione and NADPH. Incubations were carried out at 28°C for 45 minutes before termination with ice-cold 2 M acetic acid and internal standard AFG₁.

Statistical analysis

Statistical analysis was performed with SAS, version 6.10 (SAS Institute Inc., 1989). Differences in the means between two groups were determined using the t-test procedure (equal or unequal variance t-test). Time or dose response data were analyzed using analysis of variance (GLM procedure), followed by polynomial trend analysis. The dietary β NF study was analyzed using two way analysis of variance (days and β NF dose effects). A p value less than 0.05 was considered significant in all analyses.

Results

Investigation of CYP1A induction in adult zebrafish by Aroclor 1254, β NF, and TCDD

The dietary exposure of adult zebrafish to 500 ppm Aroclor 1254 for up to 9 days failed to increase CYP1A protein quantities above control levels, and did not measurably induce hepatic EROD activity. A subsequent study investigating the induction of CYP1A following i.p. injection of 100 or 200 mg Aroclor 1254/kg body weight also showed no response to this common inducer. A second attempt to induce CYP1A following i.p. injection of 75 or 150 mg Aroclor 1254 was again unsuccessful. Thus Aroclor 1254 failed to evoke a detectable response in the zebrafish by either dietary or i.p. treatment.

By contrast, a time-course investigation of CYP1A induction following i.p. injection of 150 mg β NF/kg body weight showed strong and maximal induction of both CYP1A protein and EROD activity by 24 hours, the first time-point examined following injection, with a curvilinear decrease over time (linear, $p=0.0001$; quadratic, $p<0.004$) (Fig. 1, panel A). There was a rapid decrease in both protein and activity during the remaining course of the week, with barely detectable levels of EROD activity and non-detectable levels of protein at day 7. A dietary exposure of 500 ppm β NF for 3 or 7 days demonstrated an approximate 3-fold increase in protein levels and EROD activity above controls ($p<0.04$, β NF main effect), but there was no statistical difference between the days ($p=0.7$) (Fig. 1, panel B). Noteworthy is that low but detectable levels of CYP1A were observed in the controls from the dietary exposure, whereas control values in the injection experiment were non-detectable. This is suggestive of a dietary component possessing some CYP1A-inducing activity.

Additional studies were conducted to further investigate the response of zebrafish CYP1A following exposure to β NF. Another time-course experiment using a narrower range of time was performed, because CYP1A was already maximally induced at the first time point examined in the previous time-course experiment. This study showed that protein levels displayed an increasing linear response over time ($p=0.003$), and were highest between 24 and 36 hours following injection, while the

EROD activity exhibited a significant curved response (quadratic, $p=0.035$), with an observed peak at 16 hours (Fig. 2, panel A). It is interesting to note that the amount of induction documented in this experiment is considerably less than that observed in the first study. It should also be noted that these samples showed high variability between groups, especially at the 36 hour time-point, which might explain the discrepancy with maximal induction of EROD activity occurring before peak induction of protein. Intraperitoneal injection of 25, 50, 100, or 150 mg β NF/kg body weight provided dose-responsive induction of protein and EROD activity (Fig. 2, panel B). The response to β NF was also investigated by conducting another time-course experiment following i.p. injection of 150 mg/kg body weight of β NF in corn oil instead of DMSO (Fig. 2, panel C). Over the range of four days, there was a decreasing linear trend for EROD activity ($p=0.0004$), with an observed peak at 36 hours. This time point, however, was not statistically different from the 24 hour time point ($p=0.1$), which is consistent with the experiments using DMSO as a carrier. As in the experiment in panel A, levels of CYP1A protein showed less dramatic changes than EROD activity over the period examined.

TCDD, the most potent Ah receptor agonist yet described, was found to be a potent inducer of hepatic CYP1A in this species as well. Zebrafish fed 3 days with 0.75 ppm TCDD in OTD had an EROD activity approximately 17-fold higher than controls (391.4 ± 28.7 pmol/min/mg protein compared to 23.0 ± 4.7 pmol/min/mg protein). Protein levels were also greatly induced in TCDD-treated fish (95.7 ± 17.4 densitometry units versus non-detectable levels in controls).

TCDD modulation of AFB₁ metabolism in vivo

Early excretion kinetics of AFB₁ metabolites following i.p. administration of [³H]AFB₁ was assessed by measuring the amount of radioactivity recovered in the water over 24 hours. The amount of radioactivity did not significantly differ between treatment groups. Approximately $52 \pm 13\%$ of the administered radioactivity was recovered by 24 hours in the TCDD/AFB₁ treatment group, while $45 \pm$

7% was accounted for in the AFB₁ treatment group (Fig. 3). These data show that, overall, early excretion kinetics of [³H]AFB₁ were not strongly affected by preexposure of zebrafish to TCDD.

However, whereas the total amount of radioactivity recovered in the water was similar, the actual metabolic profile of AFB₁ was different between the two groups. The AFB₁ treatment group exhibited a similar metabolic profile to that seen in the earlier metabolism study (Troxel *et al.*, publication in progress). Aflatoxinol (AFL) accounted for approximately 17% of the original dose administered, followed by unreacted AFB₁ at 6.1% and aflatoxinol-glucuronide (AFL-g) at 5.3% (Fig. 4, panel A). Less than 1% of the administered dose was recovered as aflatoxin M₁ (AFM₁), aflatoxinol M₁ (AFL-M₁), or aflatoxinol-M₁-glucuronide (AFL-M₁-g) combined. As was expected in the TCDD/AFB₁ treatment group, there was a higher production of AFM₁/AFL-M₁ and of the glucuronides. AFL-M₁-glucuronide was the predominate metabolite, accounting for 8.6% of the original dose, followed by AFB₁ and AFL with approximately 7.5% each, AFM₁ and AFL-M₁ with 5.6%, and lastly AFL-g with 4.0% (Fig. 4, panel B). Treatment of the water samples with sulfatase demonstrated that sulphate conjugation represented only a minor metabolite (at most representing less than 0.5% of the administered dose) in the zebrafish.

Figure 5 shows the proportion of each metabolite recovered at each time point as a percentage of the original dose administered. Overall, the total percentage of recovery of the metabolites varied slightly between the groups, with 29% of the dose recovered in the AFB₁ group, and 33.2% in the TCDD/AFB₁ group. The slight increase in metabolites recovered in the TCDD/AFB₁ group occurred primarily at the 24 hour time point, where there was the increased production and excretion of the glucuronides of AFL and AFL-M₁. The greater production of AFL-M₁-g compared to AFL-g might reflect substrate availability, or perhaps the more polar AFL-M₁ may be preferentially glucuronidated over AFL.

TCDD modulation of in vivo hepatic AFB-DNA adduction and in vitro activation of AFB₁ and AFM₁

The *in vivo* hepatic AFB-DNA adduction at 24 hours after i.p. injection of 400 μg [^3H]AFB₁/kg body weight was determined to be almost 4-fold higher in the group pre-exposed to TCDD than in the control group (1660 ± 175 pmol AFB₁/mg DNA versus 438 ± 109 pmol AFB₁/mg DNA, $p < 0.05$) (Table 1). To further investigate the cause of this notable difference in adduction levels, *in vitro* AFB₁ and AFM₁ metabolism studies were conducted (Table 1). The results from these metabolism experiments showed no statistical difference between the control or TCDD-treated groups in their ability to bioactivate AFB₁ or AFM₁ to the respective reactive intermediate. There was also no statistical difference between the two groups in their capability of metabolizing AFB₁ to the primary metabolite AFL. However, the TCDD-treated group did have a 22-fold increase ($p=0.0001$) in their capability to metabolize AFB₁ to AFM₁, a reaction believed to be mediated by CYP1A in zebrafish as has been shown for trout (You *et al.*, unpublished results).

Effects of TCDD on CYP1A protein and EROD activity.

CYP1A protein levels and EROD activity were measured at day 0 (the time that the AFB₁ dosage was administered) in eight control and eight TCDD-treated fish to provide a representative value of CYP1A induction in the zebrafish at the time of AFB₁ dosing. The results were in accordance with the preliminary TCDD-dietary exposure already described earlier in this section. The average EROD activity was 337.2 ± 10.4 pmol/min/mg protein, and protein levels were 127.8 ± 4.1 densitometry units. In this experiment, no CYP1A activity was detectable in the controls. CYP1A induction was also measured in the supernatant from the samples used in the DNA-adduction and metabolism experiments. Protein levels and EROD activity were non-detectable in the samples that received no TCDD treatment (i.e., the groups fed control diet and injected with either DMSO or [^3H]AFB₁). CYP1A protein levels and EROD activity were comparable between the TCDD-treated groups (i.e., the groups fed TCDD and injected with

DMSO or [^3H]AFB $_1$). The TCDD-treated group that received AFB $_1$ had a 1.6-fold higher mean EROD activity than the TCDD-treated group injected with carrier only (550 ± 64.4 pmol/min/mg protein versus 344 ± 68.4 pmol/min/mg protein), but this difference in mean activity did not achieve significance ($p=0.07$) (Fig. 6).

Discussion

CYP1A in zebrafish

A cytochrome P4501A-like protein has been found in all fish species so far investigated, including zebrafish. Using anti-trout P4501A1 IgG, Buchmann *et al.* (1993) noted a single cross-reactive band from zebrafish liver microsomes following dietary exposure to TCDD, and Collodi *et al.* (1994) found 2 cross-reactive bands (50,000 and 54,000 kDa) from zebrafish liver homogenates following exposure to a static water bath containing TCDD. Our results showed that a single cross-reacting band was induced in zebrafish liver homogenates following exposure to β NF or TCDD using this same antibody. Interestingly, both an *in vivo* static bath exposure of zebrafish to 50 μ g/l of β NF for 48 hours and zebrafish liver cells exposed to β NF *in vitro* showed no detectable induction of a CYP1A-like protein (Collodi *et al.*, 1994; Miranda *et al.*, 1993). Our studies, however, demonstrated that zebrafish CYP1A was indeed responsive to this flavone in the whole animal following i.p. administration or dietary exposure. The explanation for this difference is unknown.

β NF induction of CYP1A

Induction of the CYP1A-like protein and associated EROD activity following exposure to β NF is similar to that seen in other fish species, including the rainbow trout, Japanese medaka, and goldfish (Zhang *et al.*, 1990; Schell *et al.*, 1987; Gooch and Matsumura, 1983). The decreased levels of activity seen between the first and second time-course experiments is not too surprising. While both experiments were conducted using fish from the same spawning, the experiments were not performed concurrently. The levels and inducibility of CYP1A in fish can vary greatly depending on the reproductive status of the fish (Förlin and Haux, 1990; Larsen *et al.*, 1992). Courtenay *et al.* (1994) investigated CYP1A mRNA expression in Atlantic tomcod and found varying degrees of CYP1A mRNA expression in

prespawning, spawning, and spent male and female tomcod. The consequences of continuous spawning in such fish as the zebrafish are not known at this time.

Lack of Aroclor 1254 induction of CYP1A

The lack of induction of CYP1A in zebrafish liver following both dietary exposure and i.p. administration of Aroclor 1254 is puzzling. This commercial mixture of PCBs containing 54% chlorine is usually quite effective as a 3-methylcholanthrene-type inducer in most species. Only seldom is non-responsiveness to this mixture documented. One example is the study by Yawetz *et al.* (1992), in which exposure of a Mediterranean mollusc species to Aroclor 1254 increased overall P450 levels in the digestive gland, but actually decreased EROD activity. In another example, redfish receiving a single i.p. injection of Aroclor 1254 did not show any increase in microsomal mixed function oxidases (Stahl *et al.*, 1984). Studies in fish using high doses of β NF, benzo[a]pyrene, or planar PCBs have demonstrated that high doses of these inducers can actually lead to inhibition of activity or mRNA of the very protein being induced (Gooch *et al.*, 1989; Goddard *et al.*, 1987; Haasch *et al.*, 1993; Melancon and Lech, 1983). In the present study, not only was EROD activity non-detectable, but protein levels were also not induced above control values, even at the lowest dose (75 mg/kg body weight) investigated. Another possible explanation might be that induction by this PCB mixture could be delayed, and simply was not detected by 24 hours following i.p. administration. The dietary exposure, however, was continued for 9 days with continuous feeding, and was also unsuccessful at inducing CYP1A. The Ah receptor has not yet been isolated in zebrafish. While the assumption is that the mechanism of induction of CYP1A in zebrafish is mediated by the Ah receptor, this has not been verified. If the Ah receptor is indeed present, it might be that the agonist binding site is more selective in this species.

TCDD modulation of AFB₁ metabolism

The *in vivo* metabolism of AFB₁ in zebrafish observed in this experiment was similar to the metabolic profile documented in a previous study (Troxel *et al.*, Chapter 2 of this thesis). The predominant metabolites excreted into water over 24 hours were again AFL, AFB₁, and AFL-g. The AFB₁ metabolites excreted by the TCDD pre-treated group consisted of AFL, AFB₁, and AFL-g, but additionally included AFM₁/AFL-M₁ and the glucuronide of AFL-M₁. It is believed that CYP1A1 is the enzyme responsible for production of AFM₁, and is postulated that AFM₁ can become further hydroxylated to form AFL-M₁, which can then be glucuronidated, as is the case in rainbow trout (Loveland *et al.*, 1983; 1984; Goeger, *et al.*, 1988; You, publication in progress). Therefore it is no surprise to see increased levels of AFM₁/AFL-M₁ and the glucuronide of AFL-M₁.

The *in vitro* metabolism experiments showed increased formation of AFM₁ in the TCDD-treated liver homogenates, confirming the results of the *in vivo* study. Although there was a significant difference in the *in vivo* production of AFL between the control and TCDD-treated groups (17.1% versus 7.5%), there was no difference in the formation of AFL between the two groups in the *in vitro* metabolism assay. TCDD-treatment also did not affect the ability of the liver homogenates to bioactivate AFB₁ and AFM₁ *in vitro*. Unfortunately, enzyme activities could not be calculated in terms of P450 content, but rather only on a per mg protein basis, since zebrafish livers were too small to permit total P450 quantification. Analysis of CYP1A in the supernatant from zebrafish liver homogenates used in the metabolism and DNA adduction experiments verified that this protein and its associated EROD activity were induced by the TCDD treatment.

TCDD modulation of AFB₁-DNA adduction

The results from the investigation of TCDD modulation of hepatic AFB₁-DNA adduction showed an approximate 4-fold increase in the amount of hepatic DNA-adduction in the TCDD-treated group. A

study by Walsh *et al.* (1992) also demonstrated that TCDD pretreatment increased DNA adduction and toxicity following exposure to AFB₁ in a human epidermal cell line. The increased binding in the TCDD-treated group of zebrafish cannot be explained by an increased ability to bioactivate AFB₁ to the reactive 8-9-epoxide, since the *in vitro* AFB₁ metabolism assay failed to show any difference in this activity between groups. The increased binding could be related to the increased production of AFM₁ and/or AFL-M₁, and further activation to a DNA binding species. The *in vitro* AFM₁ metabolism assay did demonstrate that zebrafish are quite capable of bioactivating AFM₁ to a reactive intermediate capable of binding glutathione and forming an adduct. *In vivo* genotoxicity tests of AFM₁ in *Drosophila melanogaster* showed a only a 3-fold lower potency for damaging DNA compared to AFB₁ in one test, and equal genotoxicity in another test (Shibahara *et al.*, 1995). Work with rainbow trout hepatocytes also demonstrated that AFM₁ and AFL-M₁ had DNA binding values approximately 80% that of AFB₁ (Loveland, *et al.*, 1988).

In conclusion, zebrafish possess a CYP1A-like protein that is readily inducible following i.p. administration or dietary exposure to β NF, but is refractory to Aroclor 1254 at the doses investigated in this study. Dietary exposure to TCDD, the most potent Ah receptor agonist, was also found to effective. Following exposure to TCDD, the metabolism of AFB₁ was altered in a pattern consistent with increases in CYP1A, which is usually considered a detoxifying pathway. However, this pathway appears to correlate with an increase in the toxicity of AFB₁ in this species, as measured by an approximate 4-fold increase in hepatic AFB₁-DNA adduction.

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Fig. 1. Induction of CYP1A protein and EROD activity in adult female zebrafish following i.p. or dietary administration to β NF. A) Single i.p. injection of 150 mg β NF/kg body weight (data are means \pm SEM from 3 pools of 2 fish each) B) dietary exposure to 500 ppm β NF (data are means \pm SEM from 4 pools of 2 fish each for treated, and 2 pools of 2 fish each for controls). * and # designate statistical difference ($p < 0.05$) from the observed peak for protein induction (*) and EROD levels (#).

Fig. 2. Induction of CYP1A protein and EROD activity in adult female zebrafish by β NF following different treatments. A) single i.p. administration of 150 mg β NF/kg body weight with sampling up to 36 hours after injection B) i.p. administration of 25, 50 100, or 150 mg β NF/kg body weight and sampled 24 hours later C) single i.p. administration of 150 mg/kg body weight of β NF in corn oil instead of the carrier DMSO, sampled up to 4 days following injection. All data are means \pm SEM for 4 pools of 2 fish each). * and # designate statistical difference ($p < 0.05$) from the observed peak for protein induction (*) and EROD levels (#).

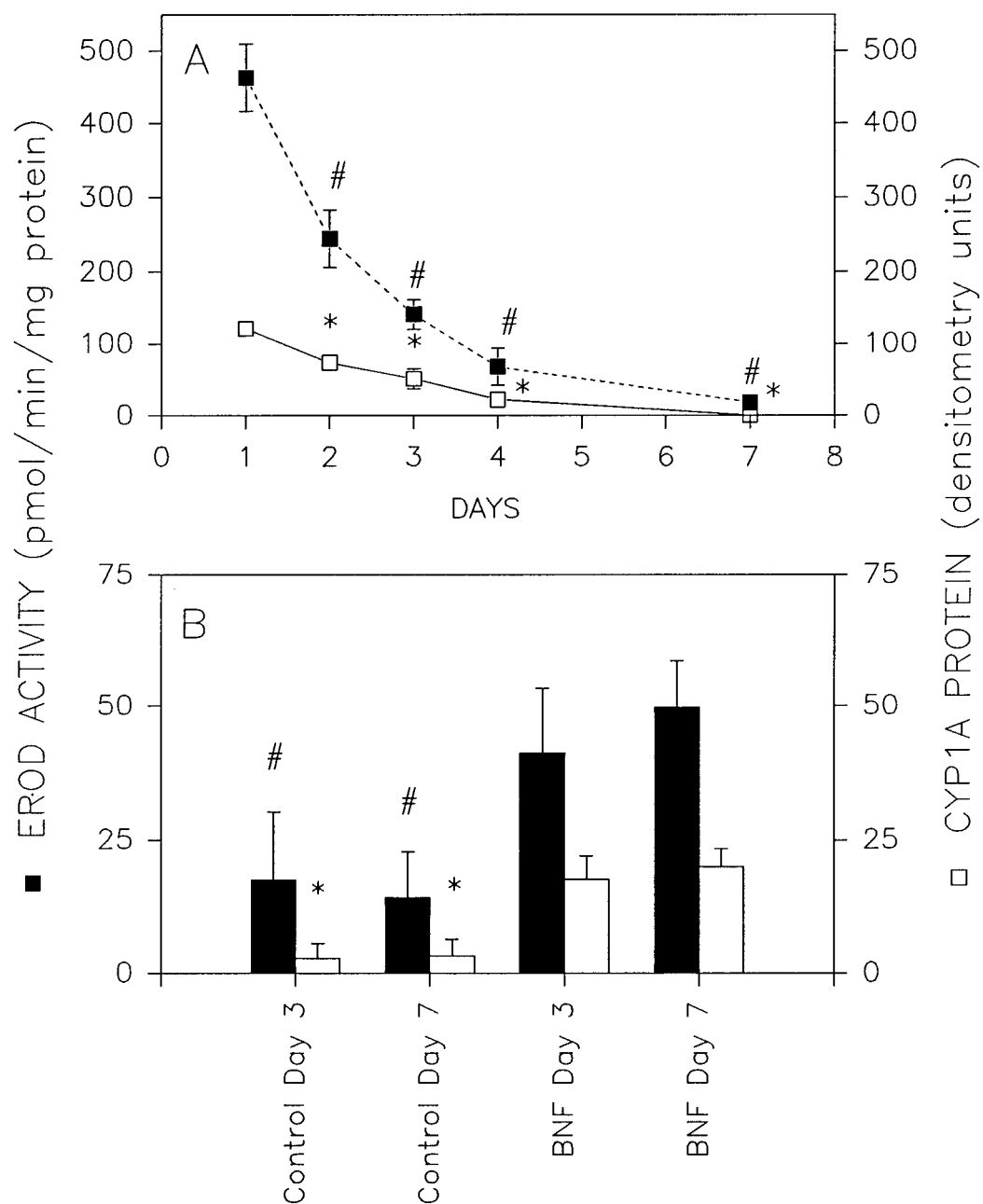
Fig. 3. The cumulative percentage of radioactivity recovered in the water within 24 hours in control and TCDD-treated zebrafish following i.p. administration of [3 H]AFB₁.

Fig. 4. The cumulative percentage of individual metabolites recovered in water over 24 hours following i.p. administration of [3 H]AFB₁, expressed as a percentage of the original dose administered A) in the control-fed group B) in the TCDD-fed group.

Fig. 5. The cumulative percentage of all metabolites recovered in water, expressed as a percentage of the original dose A) in the control-fed group B) in the TCDD-fed group.

Fig. 6. Levels of CYP1A protein and associated EROD activity in the supernatant from zebrafish liver homogenates used in the metabolism and DNA adduction experiments. (Data are means \pm SEM from 3 pools of 4 fish each, and 1 pool of 3 fish each).

Figure 1



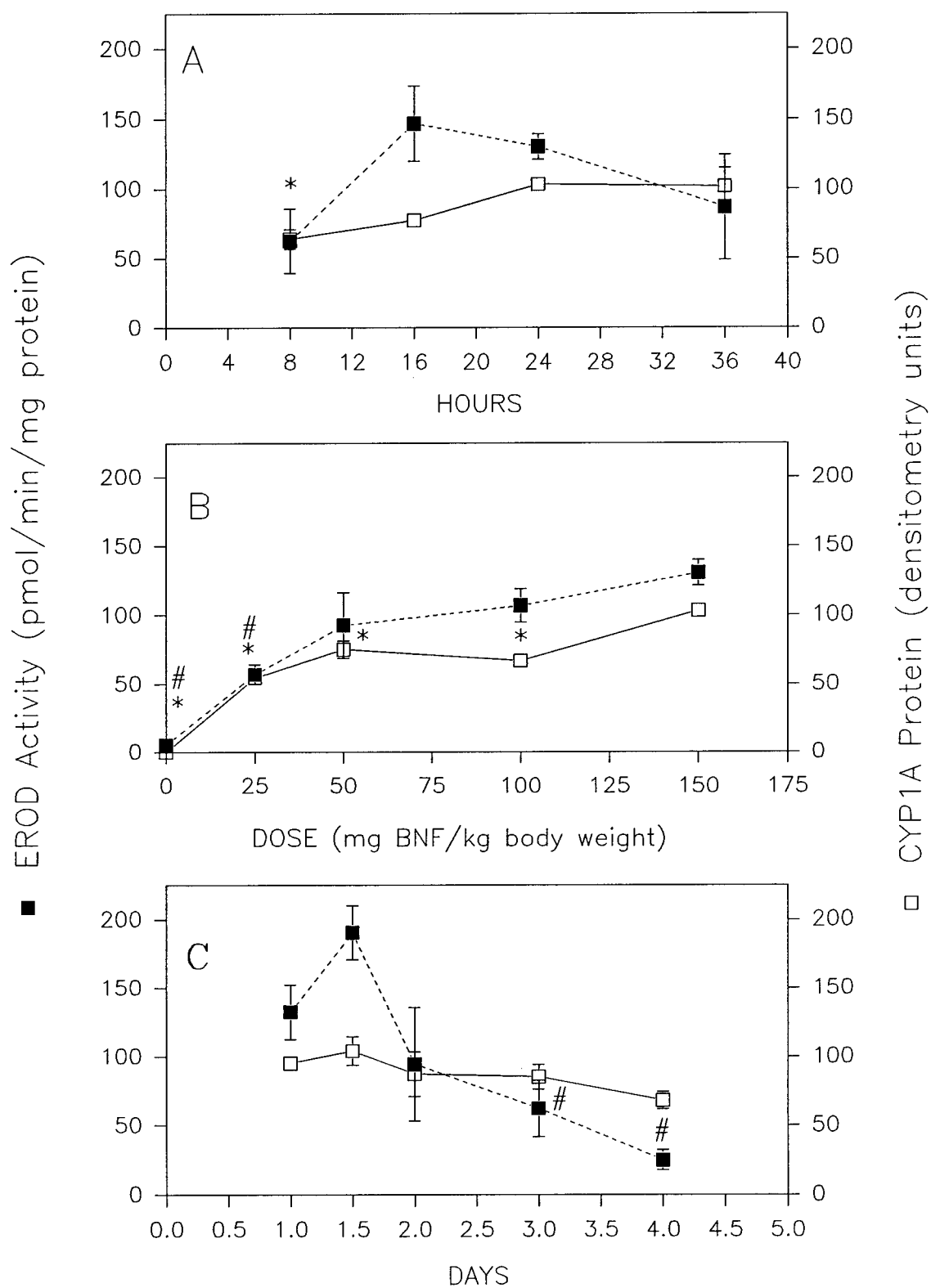


Figure 3

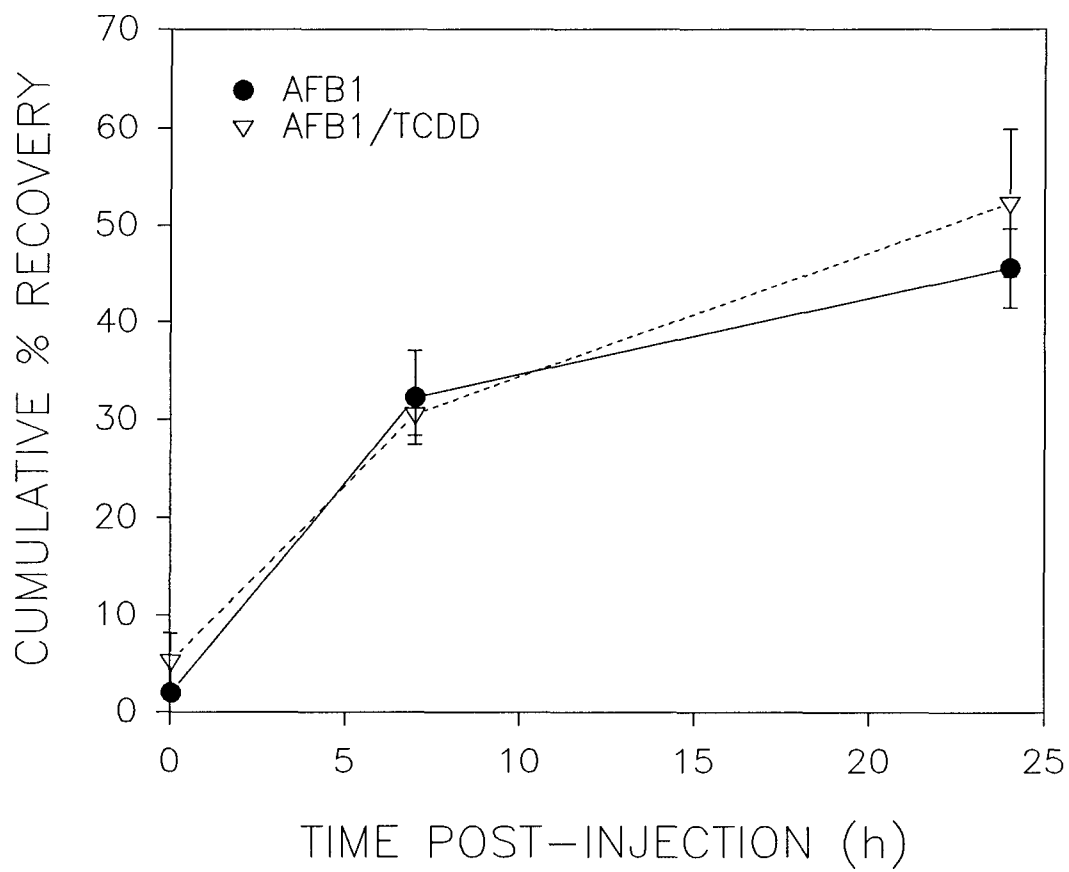


Figure 4

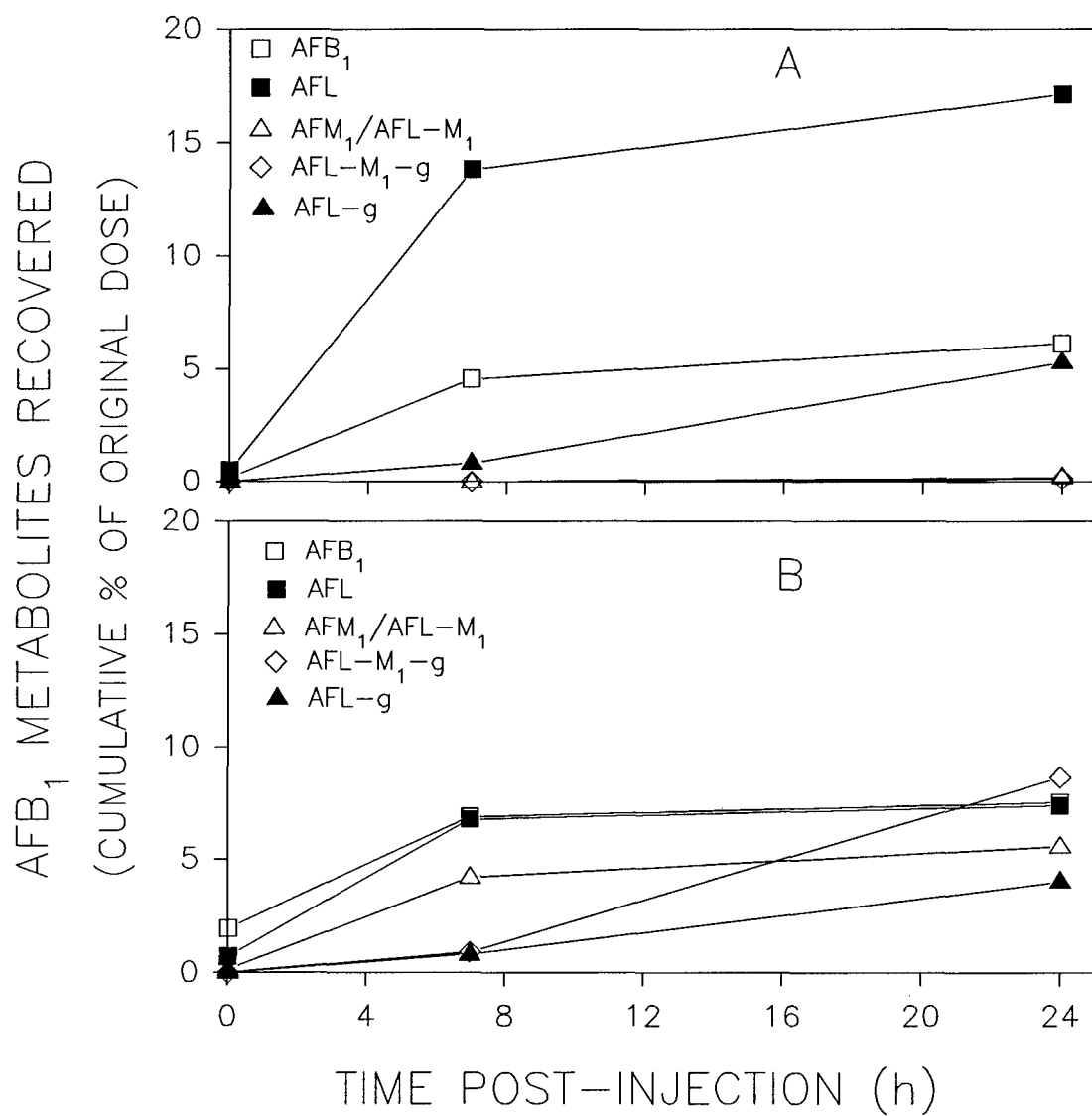


Figure 5

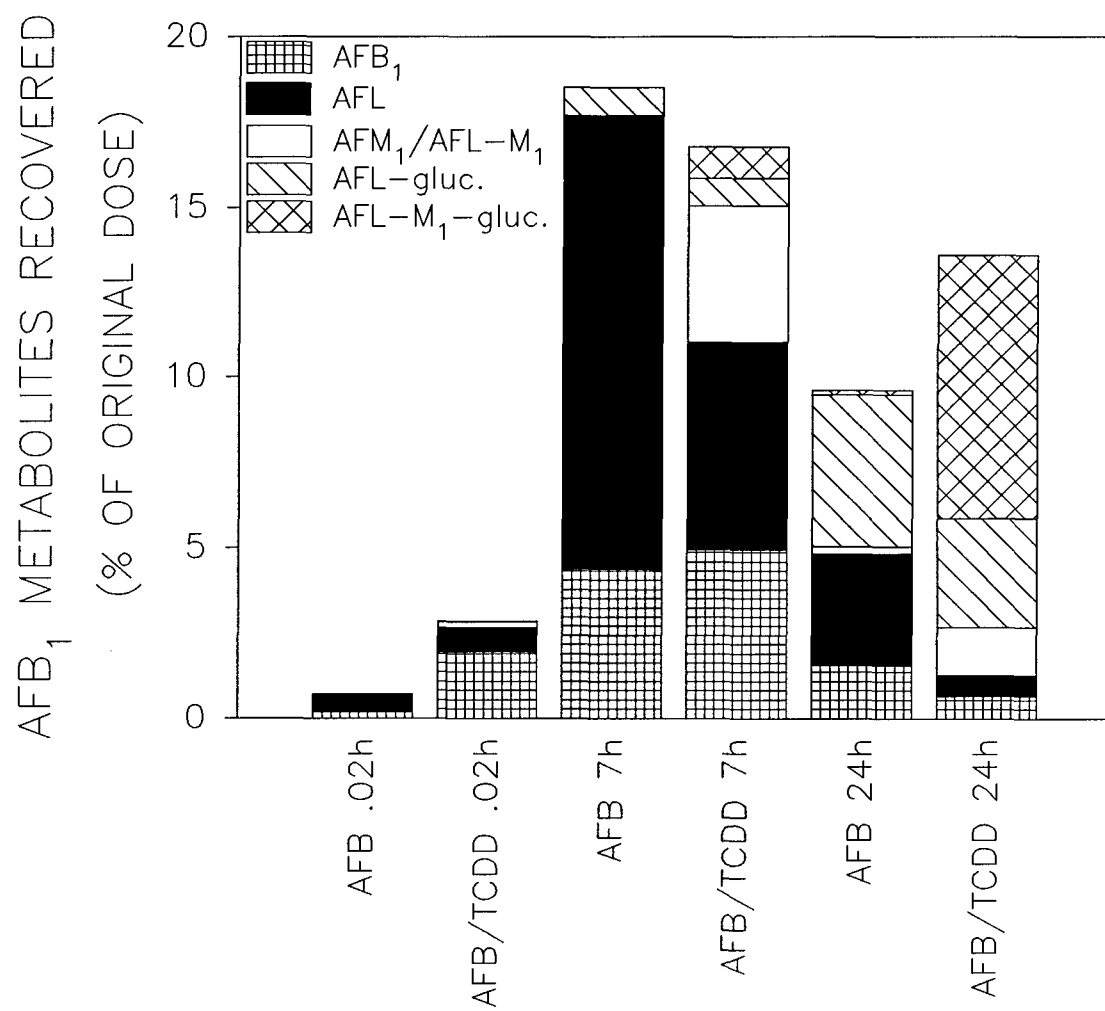


Figure 6

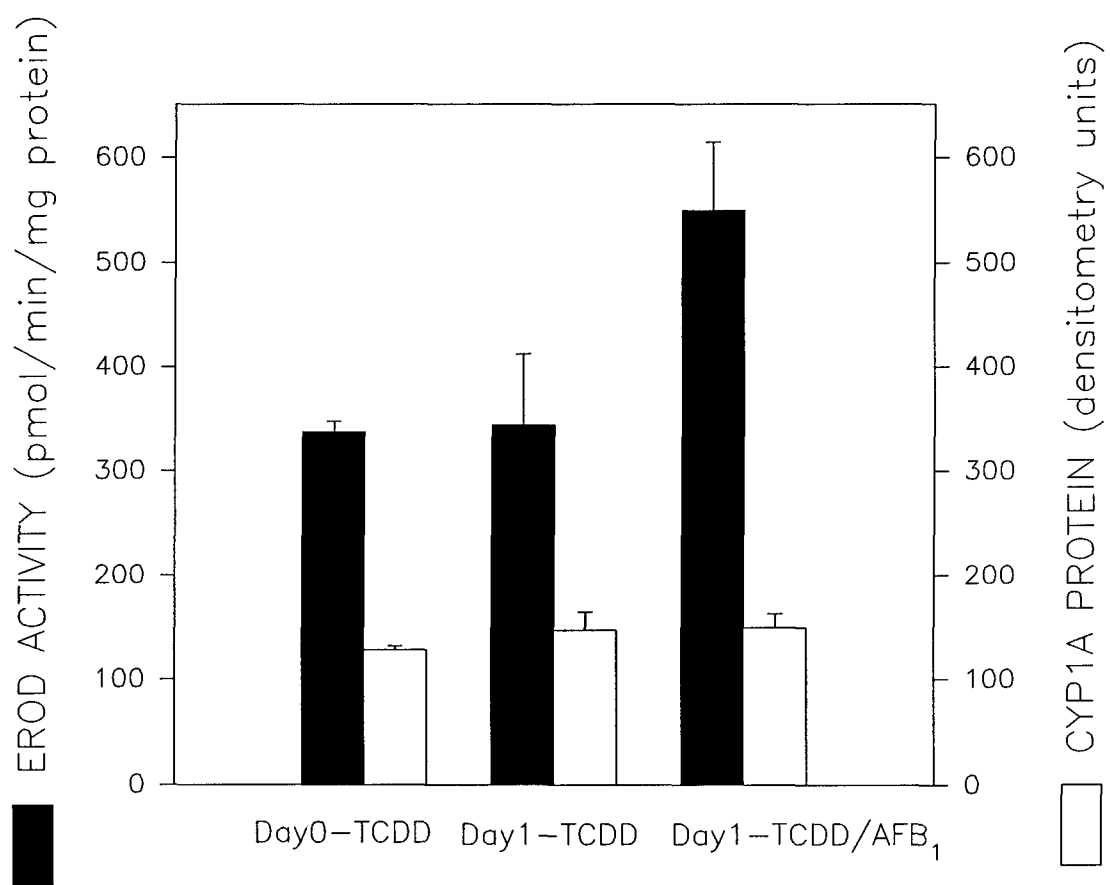


Table 1. Effects of TCDD pretreatment on *in vivo* hepatic AFB₁-DNA adduction and *in vitro* AFB₁ and AFM₁ bioactivation and metabolism^a.

	AFB ₁	TCDD/AFB ₁
^a hepatic DNA adduction		
pmol AFB ₁ /mg DNA	438.2 ± 109.1	1660.2 ± 175.4
^b aflatoxin-8-9-epoxide		
pmol/min/mg protein	13.5 ± 5.4	9.5 ± 1.0
aflatoxin M ₁ (AFM ₁)		
pmol/min/mg protein	1.0 ± 0.3	24.2 ± 3.0
aflatoxicol (AFL)		
pmol/min/mg protein	9.2 ± 2.4	8.3 ± 0.8
^c aflatoxin M ₁ -GSH adduct		
pmol/min/mg protein	8.2 ± 3.1	6.2 ± 0.6

^a data are means ± SEM from 3 pools of 4 fish each, and 1 pool of 3 fish each

^b assays were run in duplicate with a final substrate concentration of 80 μmol AFB₁, with nonenzymatic background subtracted

^c assays were run singly, with a final substrate concentration of 80 μM AFM₁, with nonenzymatic background subtracted.

Cloning, sequencing, and embryonic expression of an N-ras proto-oncogene isolated from an enriched zebrafish (*Danio rerio*) cDNA library

Ronshan Cheng, Sam Bradford¹, David Barnes¹, David Williams, Jerry Hendricks, and George Bailey²

Department of Food Science and Technology, and Department of Biochemistry and Biophysics¹, Oregon State University, Corvallis, OR 97331-6602

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²Corresponding author: telephone (503) 737-3164, FAX (503) 737-1877

Abstract

An enriched zebrafish (*Danio rerio*) cDNA library was constructed for screening of ras-related genes, and a positive clone was isolated from one plate of 3×10^4 plaques. This clone, Zras-B1, carried a 2592 bp insert with an open reading frame encoding a ras p21 protein of 188 amino acids. The deduced N-terminal 86 amino acid residues and the C-terminal CAAX binding motif are identical to mammalian ras. The full-length Zras-B1 encoded protein is most closely related to human N-ras (91% identity), with lesser homology to Ha-ras (84%) and Ki-ras (85%). Preliminary screening data also indicate additional ras genes in zebrafish, at least one of which is also transcribed in adults. A Zras-B1-related 3.1 kb transcript was found to be abundant in embryos from zygote through gastrulation, and may be maternally derived.

Introduction

There are three known ras proto-oncogenes in mammalian genomes, designated N-, Ha- and Ki-ras (Parada et al., 1982; Der et al., 1982; Taparowsky et al., 1983). These genes encode 21 KDa (p21) proteins of 188 or 189 amino acids, which undergo a number of post-translational modifications at the carboxy terminus including isoprenylation or palmitoylation essential to plasma membrane localization (Hancock et al., 1989). Ras p21 functions as a signal transduction protein (Barbacid, 1987; Bourne et al., 1990; Downward, 1992) through binding of guanosine triphosphate (GTP) and its hydrolysis to guanosine diphosphate (GDP). Evidence indicates that the GTP-bound form of p21-ras is biologically active while the GDP-bound form is inactive. The GTP-bound conformation interacts with effector molecules, and the life-span of this interaction is regulated either by the effector-mediated or the intrinsic GTPase activity of the protein. Ras-mediated signal transduction is believed to be involved in cell differentiation, development and oocyte maturation (Daar et al., 1991; Benito et al., 1991; Fortini et al., 1992). Many human and animal tumors carry ras p21 activated by point mutations at certain positions, especially at amino acid residues 12, 13, and 61, to produce acutely transforming products defective in GTPase activity and thus held in a chronically active configuration (Bos et al., 1988).

Mammalian ras homologues have been found in yeast, mollusks, and fish (DeFeo, et al., 1983; Swanson et al., 1986; Nemoto et al., 1986, 1987; McMahon et al., 1990; Mangold et al, 1991; Lee et al., 1994). However, no reports have described ras gene sequences or their involvement in differentiation or carcinogenesis in the zebrafish (*Danio rerio*).

This small tropical freshwater fish has received considerable attention as a model for vertebrate embryology and developmental biology (Streisinger et al., 1981; Kimmel, 1989), and was the first aquarium species reported to show a tumorigenic response following carcinogen treatment (Stanton, 1965), yet the molecular basis for cancer development in this species is entirely unknown. In this initial study we report the construction of a magnetically enriched whole-fish cDNA library for the isolation and characterization of ras-related sequences from zebrafish.

Results

Initial screening for zebrafish ras-like sequences in genomic DNA and mRNA

Rainbow trout Ki-ras exon 1 primers (H01 and c37) were used initially to amplify zebrafish genomic DNA by polymerase chain reaction (PCR) under low stringency conditions. PCR products were separated on 5% acrylamide gels and a DNA band migrating at the expected 110bp region was excised for cloning (data not shown). After cloning, bacterial colonies were probed by a similar PCR, and 26 of 29 colonies (93%) were found to carry 111 bp inserts. Preliminary plasmid sequencing of selected clones revealed unique ras-related sequences of 66bp located interior to the two primers. Since the ras-like sequences differed at several sites from any trout or mammalian ras-containing plasmids in our laboratory, we assumed them to be of zebrafish origin. Figure 1 shows the two categories (Z-1 and Z-5) of ras-related DNA sequence obtained from the insert-positive clones. Six out of 18 clones were Z-1 and seven out of 18 clones were Z-5 sequences. The remaining five clones had intra-primer DNA sequences unrelated to ras. The translated Z-1 sequence is identical in amino acid sequence to mammalian ras proteins within the central 22-codon region. By comparison, the Z-5 sequence contained five amino acid substitutions, three of which are not conservative (Figure 1). This sequence may derive from a related non-ras G-protein.

To obtain initial sequence information on ras-related genes that were transcribed, total adult zebrafish poly(A)⁺ mRNA was isolated and first-strand cDNAs were synthesized by reverse transcriptase. First strand cDNAs served as template for PCR reactions using HZras-08 and sc67(1)G primers (see Experimental Procedures) under the same conditions

used for genomic DNA amplification. The resulting PCR products were separated by gel electrophoresis and DNA bands migrating near the expected 200bp were excised for cloning (data not shown). Twenty-four of 29 resulting colonies gave positive PCR reactions and, by in-house plasmid sequencing, all 24 clones were the Z-1 sequence type within this 200 bp region. As seen in Figure 2, nucleotide sequencing revealed two Z-1 ras sequences (Z-RTPCR-1 and -2), differing by a single redundant nucleotide change at amino acid 34 codon, third base position. The deduced amino acid sequences of Z-RTPCR-1 and -2 were identical to mammalian ras proteins in this region. Z-RTPCR-1 was then used as a zebrafish-specific probe for the isolation of a full-length zebrafish ras cDNA.

A full-length zebrafish ras clone isolated from enriched cDNA library

Efforts using trout ras primers and probes to obtain a full-length ras clone from a traditional zebrafish cDNA library were unsuccessful (data not shown). However, the alternative strategy of constructing a cDNA library enriched for expressed ras-related zebrafish genes and using a PCR zebrafish-derived probe proved successful. To synthesize the ras-enriched cDNA library, whole zebrafish poly(A)⁺ mRNA was magnetically separated from total RNA (see Experimental Procedures), then incubated at room temperature in 50% formamide with a streptavidin-bound cZRAS-BII primer derived from Z-RTPCR-1. After cDNA synthesis from this enriched mRNA fraction, the presence of ras-related sequences was verified and the amount roughly quantified by PCR. Appropriate amounts of cDNA were ligated into the ZAP Express system for later use in plaque screening. A non-radioactive digoxigenin (DIG)-labeled probe was constructed by PCR using Z-RTPCR-1 as a template and HZras-08 and sc67(1)G as

primers. Membrane lifts from a single plate with 3×10^4 plaques were hybridized with the DIG-labeled probe followed by chemiluminescence detection. Six potential clones were isolated for second and third screenings, with two of the six showing positive results. The pBK-CMV phagemids containing the positive inserts were excised and bacterial colonies isolated. Plasmid isolation and restriction analysis indicated the sizes of these inserts, B1 and B5, were 2.6 kb and 0.6 kb, respectively (data not shown). The evidently truncated B5 insert was not further examined.

The complete nucleotide sequence of the 2592 bp Zras-B1 insert was established by the Sequenase Version 2.0 procedure and is shown in Figure 3. The deduced amino acid sequence of the Zras-B1 p21 protein is compared with mammalian ras p21 in Figure 4. Interestingly, the Zras-B1 nucleotide sequence differed in the amino acid residue 8-60 overlap region from both of the RT-PCR fragments obtained by initial screening (Figure 2). Differences were observed in 11 positions, 9 of which were redundant and 2 were conservative (Leu to Ile, codon 19); the number and redundancy of these many differences does not suggest that they could have arisen through reverse transcriptase or PCR polymerase errors. This result provides a preliminary indication that several ras-related proteins are expressed in the zebrafish. Additional studies would be necessary to establish if these may be orthologues of mammalian Ki- or Ha-ras genes.

Z-ras expression in zebrafish embryos

Northern blot hybridization analysis was carried out on total RNA isolated from synchronously-developing zebrafish embryos (maintained at 28°C) <1, 3, 6, 9, 12, 24, and 48 hours post-fertilization. A single transcript band of approximately 3.1 kb was

identified using the Zras-B1 insert as probe (Figure 5). This transcript was most abundant in early embryos from the zygote stage through gastrulation (< 1-6 hours), and thereafter gradually declined to barely detectable levels at 48 hours. The Zras-related mRNA detected in samples taken soon after fertilization is most likely maternally-derived, since activation of zygotic transcription is a characteristic of the midblastula transition (Kimmel, 1989), the onset of which does not occur until cell cleavage 10 (between 2.5 and 3 hours post-fertilization at 28.5°C) (Kane and Kimmel, 1993).

Shown for comparison in Figure 5 are Northern hybridization analyses of the same RNA samples using as probes zL-myc, which was also present in maternally-derived mRNA, and zN-myc, which was not detectable prior to the onset of zygotic transcription (Schreiber-Agus et al., 1993). Also shown in Figure 5 is RT-PCR analysis indicating the temporal expression pattern of the zebrafish homologue of the murine *T* gene (*z-T*). As previously reported (SchulteMerker et al., 1992), *z-T* was first detectable as a very faint signal 3 hours post-fertilization but not prior to the midblastula transition.

Discussion

Ras cloning

The techniques used to isolate ras-related genes have been reviewed by Chardin (1993). Methodologies based on traditional poly(A)⁺ mRNA cDNA library synthesis without specific enrichment of pertinent sequences often prove difficult for efficient isolation of mRNAs of low copy number. Magnetic enrichment can assist in isolating low copy messengers from complex cDNA or genomic libraries (Morgan et al., 1992), and also offers an alternative to isolate sequence-related genes such as the ras superfamily from the same library. We were unsuccessful in attempts to identify ras-related clones from an unenriched zebrafish cDNA library using trout-derived probes and primers. By comparison, use of zebrafish-derived probes and an enriched library provided a full-length ras transcript from the first plate (3 x 10⁴ plaques) screened.

Homology to mammalian ras genes

The first 86 amino acids of Zras-B1 are identical in sequence to mammalian ras proteins, and the CaaX motif for C-terminal modification is maintained (Figure 4). The segment encompassing amino acids 87 to 164 is more divergent among mammalian ras proteins, and region 165-189 is hypervariable. Zras-B1 is 88, 83, and 86% identical to mammalian N-, Ha-, and Ki-ras proteins, respectively, within region 87-164, while homologies in the hypervariable c-terminal region are 64, 32, and 28%, respectively (Figure 4). Overall, Zras-B1 is 91, 84, and 85% homologous to human N-, Ha-, and Ki-ras p21, respectively. Thus, by simple amino acid sequence comparison zebrafish Zras-B1 shows closest identity to mammalian N-ras and is tentatively identified as an N-

ras. We stress, however, the precise differences that confer biologically significant properties unique to N-, Ha-, and Ki-ras p21 are not known, and it is thus not possible to conclude that the protein encoded by Zras-B1 functions in zebrafish as an N-ras protein.

Zras-B1 and the two initially isolated RT-PCR fragments share extensive nucleic acid sequence homology, with minor differences in the wobble base (Figure 2). Since the ras-related mRNAs were enriched under low stringency conditions, it is not unexpected that several related nucleotide sequences would be present in the enriched cDNA library. This result is consistent with the known ras superfamily diversity in other species, and indicates an analogous existence of multiple forms of ras-like proteins in the zebrafish.

The functioning of these proteins in zebrafish cellular differentiation, embryogenesis, and tumorigenesis remain to be established. The PCR techniques and enriched cDNA library application used here offer a relatively efficient way to search for additional ras-related genes in zebrafish.

Embryonic expression

Northern blot hybridization analysis employing the Zras-B1 cDNA insert as a probe revealed the presence of a single Zras-B1-related transcript band at 3.1 kb in total RNA isolated from early zebrafish embryos. This transcript was most abundant during the first 6 hours of embryonic development, after which it declined over the course of the first 48 hours to barely detectable levels. The presence of the transcript within one hour following fertilization indicates that it is contributed at least in part maternally. Although a single Zras-B1-related transcript band was detected in the present study, the number

of transcripts within this band and the total number of ras-related proteins expressed in the zebrafish remains unresolved. It is possible that multiple transcripts of differing molecular size might be identified under less stringent hybridization conditions than those employed here.

Previous investigators have interpreted stage-specific and tissue-related expression patterns of cellular oncogenes, including c-ras, as evidence for involvement in normal cellular proliferation and differentiation during embryonic and fetal development of the mouse (Muller et al., 1982; Slamon and Cline, 1984). The c-ras protein (p21) is specifically expressed in early and late mouse blastocysts, and monoclonal antibodies directed against p21 inhibit preimplantation embryonic development (Ahmad and Naz, 1993). Among lower vertebrates, antibodies to the ras gene product inhibit adenylate cyclase and accelerate steroid-induced cell division in *Xenopus* oocytes (Sadler et al., 1986). More recently, ras p21 has been implicated in the transduction of a mesoderm-inductive signal in *Xenopus* (Whitman and Melton, 1992). In the present study we have demonstrated the presence of a ras-related transcript in early zebrafish embryos; it is possible that a ras-related protein is involved in signal transduction pathways necessary for successful development of the zebrafish embryo.

Experimental Procedures

Reagents

Sodium chloride, citric acid trisodium salt, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, formamide, urea, boric acid, TRIZMA hydrochloride, and ammonium persulfate were purchased from Sigma (St. Louis, MO). RNase cocktail was from 5' to 3' (Boulder, CO). Acrylamide/bis-acrylamide solution, saturated phenol/chloroform, and chloroform were from AMRESCO (Solon, OH). X-OMAT AR film was from Kodak (Rochester, NY), ³⁵S-dATP was from NEN (Boston, MA), and proteinase K was from Boehringer Mannheim (Indianapolis, IN).

RNA and DNA isolation

Whole zebrafish reared at the Oregon State University Food Toxicology and Nutrition Lab, were cut into small pieces and frozen immediately in liquid nitrogen. For DNA isolation, 0.1g of ground tissue was added per 1.2ml of lysis buffer (0.1M NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS, and 0.1mg/ml of proteinase K) and incubated overnight at 37°C. The DNA was extracted once each with phenol/chloroform and chloroform, and precipitated with 2.5 volume of 95% ethanol and a final NaCl concentration of 0.2M. The DNA pellets were dissolved in TE buffer and treated with RNase (Sambrook et al., 1989). For RNA isolation, the ground zebrafish tissue was homogenized in TRIzol reagents (GIBCO BRL, Gaithersburg, MD) and the company's protocol was followed for total RNA isolation. The concentrations of RNA and DNA were determined by spectrophotometry. Magnetic separation for mRNA isolation was carried out using a Biomag mRNA purification kit following the manufacturer's protocol (Perseptive

Diagnostics, Cambridge, MA). The first strand cDNA was synthesized by Superscript preamplification system (GIBCO BRL, Gaithersburg, MD) and used for RT-PCR reaction without further purification.

Amplification Primers

The rainbow trout Ki-ras H01 primer (a forward primer located at the 5' end of exon 1; 5'- ATG ACG GAA TAC AAG CTG-3') the c37 primer (a reverse primer located at the 3' end of exon 1; 5'- CTC GAT GGT GGG GTC ATA TT-3'); the sc67(1)G primer (a reverse primer in exon 2; 5'- CAT GGC GCT GTA CTC CTC CTG-3'); and the HZras-08 primer (a zebrafish ras exon 1 forward primer; 5'- TCG TGG GAG CTG GAG GCG TA-3') were synthesized by the Center for Gene Research and Biotechnology, Oregon State University. A 5' biotinylated cZRAS-BII primer (complementary to the zebrafish Z-1 type ras mRNA exon 1 sequence; 5'- GTG AGA GCG CTT TTG CCT ACG CCT CC- 3'), was purchased from Amifotech (Boston, MA). All primers were dissolved in water without any further purification. The primer concentrations used for PCR amplification were the same as described by Fong et al. (1993).

Amplification, cloning and sequencing

The zebrafish genomic ras-like DNA sequence was amplified by PCR using Replitherm thermostable DNA polymerase (Epicentre Technologies, Madison, WI) and rainbow trout Ki-ras exon 1 H01 and c37 primers. PCR reaction conditions were as follows: one cycle of denaturing at 95°C for 6 min; 2 cycles of denaturing at 95°C for 2 min, annealing at 55°C for 45 seconds, and extension at 74°C for 25 seconds; 40 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at

74°C for 25 seconds. RT-PCR reactions for zebrafish ras exon 1 and part of the exon 2 region were also carried out using HZras-08 and sc67(1)G primers and first strand cDNA as templates. PCR cycles were identical to the above conditions. The PCR products were purified through a Microcon-30 column (Amicon Division, Beverly, MA) to remove excess primer and dNTP. Genomic PCR and RT-PCR cloning reactions followed the protocol of Invitrogen (San Diego, CA) TA cloning kit and finally transformed to *E. coli* competent cells. To screen positive clones, traces of single bacterial colonies were picked from plates, suspended in Replitherm reaction buffer and directly amplified by PCR with the same primers used for amplification. The results were analyzed by gel electrophoresis, and positive clones were isolated and recultured in LB broth with appropriate antibiotics for plasmid isolation. Plasmid sequencing reactions were carried out as specified by Sequenase Version 2.0 (USB, Cleveland, OH), and final products were resolved on 5% acrylamide denaturing sequencing gels with ³⁵S-dATP as described elsewhere (Fong et al., 1993)

Magnetically enriched ras cDNA library construction

About 900 µg of total RNA was used for total mRNA isolation by magnetic separation as above. The mRNA preparations were resuspended in 180 µl of 0.5M NaCl and 50% formamide, then incubated with cZRAS-BII primer-bound streptavidin beads at room temperature (low stringency) to enrich ras mRNA by following the manufacturer's protocol (Biomag Streptavidin system, Perseptive Diagnostics, Cambridge, MA). The beads, containing ras-enriched mRNAs, were magnetically separated, and heat eluted in diethylpyrocarbonate water. The mRNA concentrations were approximated with a DNA DipStick (San Diego, CA), and approximately 5 µg was taken for precipitation in 0.1

volumes of 3M sodium acetate and 2.5 volumes of 95 % ethanol at -70°C overnight. This material was used for library construction with the ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA) by the manufacturer's protocol.

Plaque hybridization and chemiluminescent detection

An RT-PCR clone with a 180bp zebrafish exon 1-exon 2 insert (obtained by PCR amplification with HZras-08 and sc67(1)G primers) was used as a template and nonradioactive probes were prepared by incorporating digoxigenin (DIG)-dUTP (Boehringer Mannheim, Indianapolis, IN) and other dNTPs into the PCR products under the same conditions used above. The plaque-lift hybridization methods were described by Sambrook et al., 1989. Lumi-Phos 530, a chemiluminescent formulation for alkaline phosphatase detection when using nonradioactive nucleic acid labeling, was used in cDNA library screening. The detection procedures followed the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN), briefly described as follows: duplicate membrane transfers from a single plate of 3×10^4 plaques were hybridized with DIG probe in 7% SDS, 5x SSC, 2% blocking agent, 0.1% N-laureyl sarcosine 50mM and sodium phosphate pH 6.8 solution. After blocking, membranes were incubated with anti-DIG Fab fragments conjugated to alkaline phosphatase, reacted with Lumi-Phos 530 for 30 min. at 37°C, and finally exposed to X-OMAT AR film to record the chemiluminescent signal.

Probes for Northern blot hybridization analyses

Zras-B1 (2.6 kb) cDNA was isolated from the pBK-CMV phagemid following *EcoRI/XhoI* digestion. Zebrafish *myc* probes consisted of a 560-bp *XmnI/EcoRI* fragment

specific for zN-myc and a 1.4 kb *EcoRI* fragment specific for zL-myc, as described (Schreiber-Agus et al., 1993). Digests were electrophoresed on a 1% agarose gel; inserts were visualized by ethidium bromide fluorescence, excised from the gel, and recovered using a commercial GENECLAN kit (BIO 101, La Jolla, CA) according to the manufacturer's instructions. ^{32}P -dATP-/ ^{32}P -dCTP-labelled probes were prepared by random primed labelling.

Preparation and analysis of RNA for Northern hybridization

Total RNA was isolated from wild-type zebrafish embryos (maintained at 28°C) at various times post-fertilization by the guanidinium thiocyanate method (Kingston et al., 1990). Samples of total RNA (18 µg/lane) were fractionated by electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes. Hybridizations were carried out for 17 hr at 42°C in the presence of 50% formamide, 5X Denhardt's, 5X SSC, 0.1% SDS, and 250 µg/ml sonicated calf thymus DNA.

RT-PCR for the zebrafish homologue of the murine T gene

RT-PCR was carried out using a commercial reverse transcription system (Promega) according to the manufacturer's instructions. One µg of total RNA was prepared in a 20 µl reaction mixture containing 5 mM MgCl₂, 1X RT buffer, 1 mM dNTPs, 1 U/ml ribonuclease inhibitor, 16 U AMV reverse transcriptase, and 0.5 µg oligo(dT)₁₅ primer. After incubation at 42°C for 30 minutes, 5 µl of this mixture were amplified in a total volume of 0.1 ml containing 1.75 mM MgCl₂, 1X PCR buffer, 0.15 mM dNTPs, 2.5 U Taq polymerase (Perkin-Elmer), and 0.6 mM oligonucleotide primers. Primer sequences and amplification conditions were described previously (Schulte-Merker et

al.,1992).

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Legends to Figures

Fig 1. Nucleotide sequence comparison of rainbow trout Ki-ras exon 1 region with two types of zebrafish ras-like sequences. Zebrafish genomic DNA was amplified by rainbow trout H01 and c37 primer and DNA sequences were compared between these two primers. In-house plasmid sequencing of eighteen clones showed the Z-3, 7, 11, 13, and 19 were the same as the Z-1 type and Z-6, 8, 10, 12, 18, and 21 were the same as the Z-5 type.

Fig 2. Ras exon 1 and 2 region nucleotide sequence comparison of zebrafish RT-PCR 1 and 2 clones, Zras-B1 cDNA clone and rainbow trout Ki-ras. Zebrafish RT-PCR clones were generated using the primers HZras-08 and sc67(1)G under low stringency conditions.

Fig 3. Nucleotide sequence of Zras-B1 clone. The Zras-B1 clone open reading frame encodes 188 amino acid residues. The DNA sequence has GenBank accession number U62619.

Fig 4. Deduced amino acid sequence of the Zras-B1 p21 protein compared to mammalian N-, Ha- and Ki-ras. Amino acids identical to human N-ras protein are indicated by "-", and "/" indicates a deletion.

Fig. 5. Temporal expression pattern of z-ras-related mRNA during early zebrafish

development, compared to zL-*myc*, zN-*myc*, and the zebrafish homologue of the murine *T* gene (z-*T*). Analyses were carried out as described in the text. The top three panels show results obtained in Northern blot hybridization analyses using radio-labelled z-ras cDNA, zL-*myc* subclone cDNA, and zN-*myc* subclone cDNA, respectively, as probes. The bottom panel shows expression of z-*T*, as revealed by RT-PCR (total RNA from the < 1 hour time point was unavailable for this analysis; the first lane in this panel shows absence of detectable product in a control sample not containing RNA).

Figure 1

	8											
RT Ki-ras	GTG	GTG	GGG	GCA	GGA	GGT	GTG	GGC	AAG	AGC	GCG	
Z-1	--C	---	--A	--T	---	--C	--A	---	--A	---	--T	
Z-5	---	---	--C	GAC	--T	--C	--A	---	---	---	--A	

	19											
RT Ki-ras	CTC	ACC	ATC	CAG	CTC	ATT	CAG	AAC	CAC	TTT	GTG	
Z-1	---	---	---	--A	---	--C	---	---	---	---	---	
Z-5	---	--T	---	---	TTT	TTC	---	--G	ATC	---C	---	

Figure 2

	8														
RT ki-ras	GTG	GTG	GGG	GCA	GGA	GGT	GTG	GGC	AAG	AGC	GCG	CTC	ACC	ATC	
Z-RTPCR-1	--C	---	--A	--T	---	--C	--A	---	--A	---	--T	---	---	---	
Z-RTPCR-2	--C	---	--A	--T	---	--C	--A	---	--A	---	--T	---	---	---	
Zras-B1	--T	---	--A	---	---	---	--T	--G	---	---	---	TTA	--A	---	
	22														
RT ki-ras	CAG	CTC	ATT	CAG	AAC	CAC	TTT	GTG	GAT	GAA	TAT	GAC	CCC	ACC	
Z-RTPCR-1	--A	---	--C	---	---	---	---	---	---	---	---	---	--A	--T	
Z-RTPCR-2	--A	---	--C	---	---	---	---	---	---	---	---	---	--G	--T	
Zras-B1	---	---	--C	---	--T	---	---	---	---	---	---	---	---	--T	
	36														
RT ki-ras	ATC	GAG	GAC	TCG	TAC	AGG	AAG	CAG	GTG	GTG	ATT	GAT	GGG	GAG	
Z-RTPCR-1	--T	---	---	---	---	---	---	---	---	---	---	--C	--A	---	
Z-RTPCR-2	--T	---	---	---	---	---	---	---	---	---	---	--C	--A	---	
Zras-B1	--T	---	---	--T	---	---	---	---	---	---	---	--C	--C	---	
	50														
RT ki-ras	ACA	TGT	CTG	CTG	GAC	ATC	CTG	GAC	ACT	GCA	GGT				
Z-RTPCT-1	--G	---	--A	---	---	---	---	---	---	---	---				
Z-RTPCR-2	--G	---	--A	---	---	---	---	---	---	---	---				
Zras-B1	--G	---	---	---	---	---	---	---	---	---	--C				

Figure 3

GGCACGAGCG TGAATCGTGA AGTTCTGGGG AAGCTGCTTG CTGAACCTTT ATTCAAAACT TTGGCCAGAC TTGTTTAACG
GCTGTTTATA TACCTGGATT TGTGGCCTCT CTACGAATCT GGTAGCGCG GGTACGCTG CCTCCAGACG GGTGTGAAGT
GACTTTTGA GGGCTCCTCA TGGCATTATT TTGGGCGAGG GAGCAGTAAT AAGCAGAGCC CTGTTGATCA GATCAGCCAG
AGGTCGCAGA ACTGCAGTGT AAGCGGTGGT CCCTCAGGAC TGTAAG

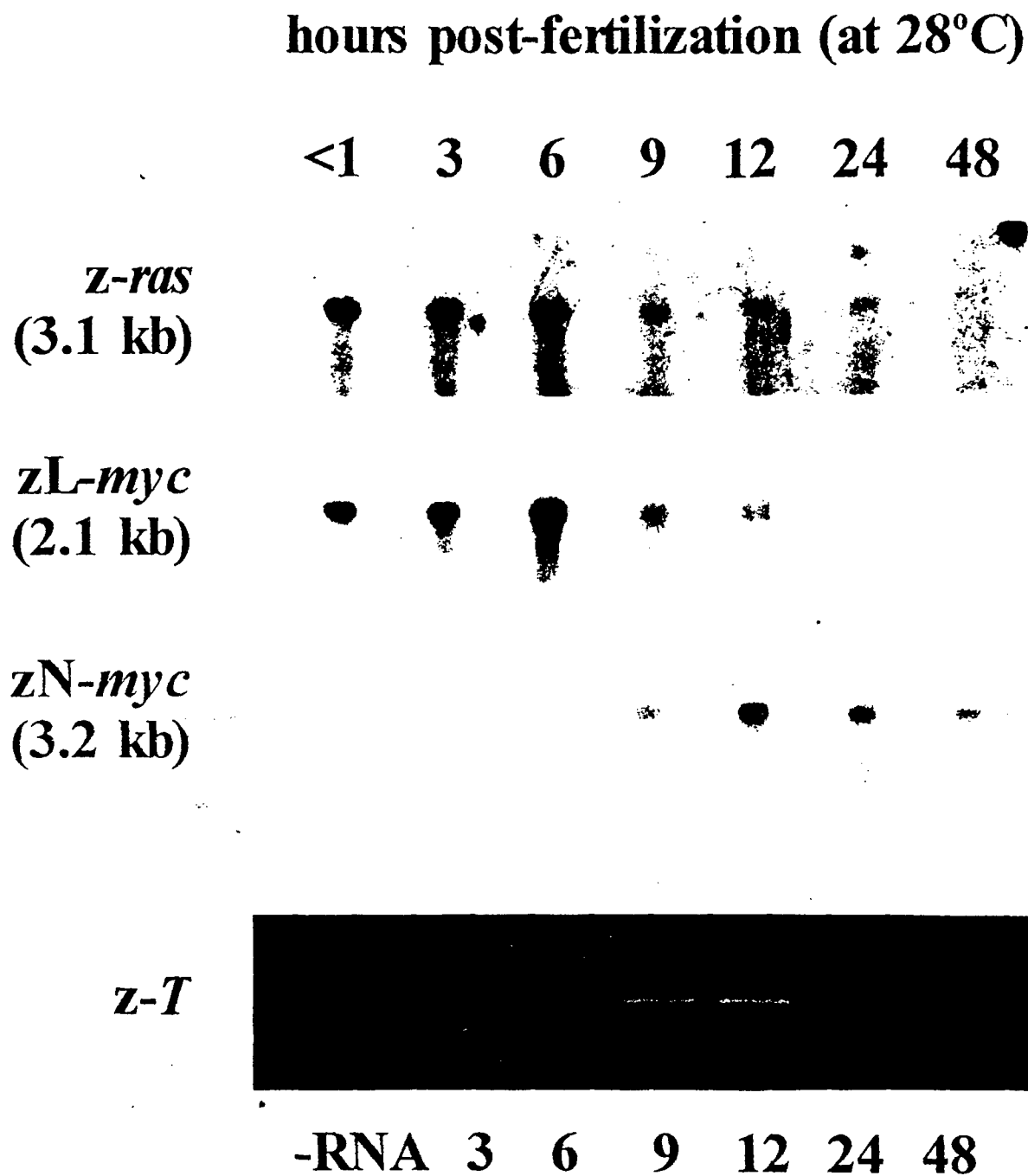
ATG ACT GAG TAT AAG CTG GTT GTT GTG GGA GCA GGA GGT GTT GGG AAG
AGC GCG TTA ACA ATC CAG CTC ATC CAG AAT CAC TTT GTG GAT GAA TAT
GAC CCC ACT ATT GAG GAC TCT TAC AGG AAG CAG GTG GTG ATT GAC GGC
GAG ACG TGT CTG CTG GAC ATC CTG GAC ACT GCA GGC CAG GAA GAG TAC
AGC GCA ATG AGA GAC CAG TAC ATG AGG ACA GGA GAG GGT TTC CTC TGC
GTC TTC GCT ATC AAC AAC AGC AAA TCC TTC GCC GAC GTG CAT TTG TAC
AGA GAG CAG ATC AAG CGT GTG AAG GAC TCG GAT GAT GTT CCC ATG GTC
CTA GTG GGG AAC ATT TGT GAT TTG GCA AGG ACT GTG GAC ACC AAG CAA
GCT CAG GAA CTT GCC AGA AGC TAC GGT ATT GAG TTT GTA GAA ACC TCT
GCC AAA ACC AGA CAG GGA GTC GAG GAT GCT TTC TAC ACC CTT GTT CGT
GAG ATC CGG CAT TAT CGC ATG AAA AAG CTC AAC AGC AGA GAA GAC AGG
AAG CAG GGC TGT CTG GGC GTG TCC TGT GAA GTC ATG TGA

CCGCACTGTC TCCCTATTTT TGTGTTTGTT TTTTGAAGC AGTTTGTTAA GCAGGCAGCT GGAAGCCTCA TGACCACCCG
TGTGCATGCG GCAGATTTTC CCCTCTGCTG GCTGAAAGGG AATAATGCAC AACTGGTCAT CTTTCCGGGT TGGCCTGCTG
TAATATATAT AAATGAGGCC GATGGACATT ACATGTTTTA ACCGAGGCTT AGTACTTAGG TTTTCTTCTC TCCTTTGGAA
GAGCAACTTA AACCGGGCAT TGAGATTGGG CATTGGATGA TTCATGCTTC TCTTCTGACC CCTTATAAAC ACGGATCAT
CAAGCAGATT GGCATTGAGG AAAATAAATA AAAATTAATA TTCTAGGCAC ATTTTATCCC AAAATCTGAA GGATTAAAT
TTTTTGTTTA TATACAATTT ATCACAATTA CCCTCATTCT TATTATAATT TAGCTATTTT TAATATCTC ATCTTTAAT
AGACATCAAA TAAAGCCATT ACCTATTACT GTGATGCATA AAAATGAATA TATTATTTCC TCATTAACAT AATGATAATT
GAGAAGGAAA TGTGCTTAAC TGCCAATCAT CATTTGACAT TAAAGCACCA CAATGGCAAA AATTCTAGAC GTATACCCAC
ATATATATAA TTTCTGATTA ATTGTTTTAA TTTTGGGATT ATATTTGGAG CTGTTCTTTG CAGGGTGTTA TGACCCGTAG
AATGTTTTAC AATACATCTG TATGTCCAGA AGAAGCTGTT TTCAAGTCAT AACTTCAGG ATTAGTATAA TTTATGACTG
TCATTTTCTC AGAATTTGTA AAGGCCTCAG AAGAGAACGA GTGAAATGCC CTTTAATCTT AATCCGCAGG TTCAATGCTC
AGGAATACTG TGAAACACAG AACTTCTTCT TCCCCAAAAG TGTGGAATTT TTCTCTTCAG TTTTTTTTTA ATTTGTTTGT
TTTCAAGGTA GCCAAAGAGT TTTAGCATT ACCATTTTTA GATACATTTG CTCTAGAGTT TCACCTTCTT TGCTCTCAGA
TGATAATTGT ATATATTGAT ATATAATACT TAATGATTG GTTCAGCTGC AGACAATTGG ATTGGAATTT AAGCATAGGG
GTTTGTGGA TTTTACTTTT AAGGGCCTTT TGAATCATGG TGCTGCTGGT GTTTATGCCA AATCTGCGTG CCGCCTTCTG
CTTTAGCTCA TATCAATGTC TTAATCTTC CAACAAAAGA TGTCCTTTAT AGCATGACAT GTTTTTCTTG GAAGTGTAAC
GGATCAGAGT AAGGTTTGTT GCATTGTTTC CATGCCTGAG GTCTCTCTT AAGAGCCGCT GAAGGTTTCC CAAGAAGTTG
AAATGATCTT TCTTGTGAC TGTGCTGTTA TTTATATGGC ATTTGCTGA AACTGTGCTT GATTTCAATG AGAATGAAAT
CTGCAATGTT TCTTGTGTT TACTGCGGTT GCGTTTGGCC TTCTGTCTC ACATAACAG AGGCAAGATG TTTGCTAGAA
AATACAGACT AAGTTTGCCA TTATGTTAAC AAATCCTTTC TTTTTTTAAA CTATATAGAA ACTTGTTTTT TGTATATTTG
TTTTACCATT TTAGCACACT TTATCATTAC CACCGACTAT CTCTAATCAG AAAGTCATGT GAAAACGAGC ATTTCATGTC
TTTTGGGGGA GGGTGGGGGA CTATACATGT GTAACAGATG TGCCTACCAT ATTACAAGAA GTTTAACCAG TAAATTAATC
GGAATCTGAA AAAAAAAAAA AAAAA

Figure 4

N-ras	1	13	25	37
Z-ras	MTEYKLVVVGAG	GVGKSALTIQLI	QNHFVDEYDPTI	EDSYRKQVVIDG
Ha-ras	-----	-----	-----	-----
Ki-ras	-----	-----	-----	-----
N-ras	49	61	73	85
Z-ras	ETCLLDILDTAG	QEEYSAMRDQYM	RTGEGFLCVFAI	NNSKSFADINLY
Ha-ras	-----	-----	-----	-----VH--
Ki-ras	-----	-----	-----	--T--E--HQ--
				--T--E--HH--
N-ras	97	109	121	133
Z-ras	REQIKRVKDSDD	VPMVLVGNKCDL	PTRTVDTKQAHE	LAKSYGIPFIET
Ha-ras	-----	-----I---	/A-----Q-	--R---E-V--
Ki-ras	-----E-	-----	AA---ESR--QD	--R---Y---
			-S-----QD	--R-----
N-ras	145	157	169	181
Z-ras	SAKTRQGVEDAF	YTLVREIRQYRM	KKLNSSDDGTQG	CMGLPCVVM
Ha-ras	-----	-----H---	-----RE-RK--	-L-VS-E--
Ki-ras	-----	-----HKL	R---PPE-SGP-	--SCK--LS
		-----L	--ISKEEKTPGC	VKIKK-II-

Figure 5



Zebrafish (*Danio rerio*) p53 tumor suppressor gene: cDNA sequence and expression during embryogenesis

key words: p53 cDNA sequence, zebrafish, multiple alignment, embryonic expression, alternate splicing

**Ronshan Cheng, Bryan L. Ford, Patricia E. O'Neal, Catherine Z. Mathews,
C. Sam Bradford¹, Thananya Thongtan¹, David W. Barnes¹, Jerry D. Hendricks, and
George S. Bailey²**

Departments of Food Science and Technology, and Biochemistry and Biophysics¹, Oregon State University,
Corvallis, OR 97331-6602

²Corresponding author: Telephone (503) 737-3164, FAX (503) 737-1877

Abstract

Three methods were successively used to screen a whole-adult zebrafish cDNA library for expressed p53-like genes. The sequences of the resultant clones describe an open reading frame 1122 nt in length, with 43 and 940 bases of 5' and 3' untranslated sequence, respectively. The deduced amino acid sequence of the zebrafish p53 is 63% identical to that of trout and 48% identical to that of human p53. Two of the three zebrafish clones overlap to span the entire reported cDNA sequence and are identical in their deduced amino acid sequence over their coincident length. The third clone contains two conservative amino acid changes, as well as an inserted amino acid subsequently found to be at the junction of exons 2 and 3. Northern analysis demonstrated the zebrafish p53-related transcript to be present and most abundant in zygotes and early-cleavage embryos (<1 hr post-fertilization), thereafter declining to barely detectable levels at 48 hours. A similar temporal expression was detected for the zebrafish L-*myc*, known to be present in maternally-derived RNA, whereas zebrafish N-*myc* and the zebrafish homologue of the murine *T* gene were not detectable prior to the onset of zygotic transcription.

Introduction

The zebrafish (*Danio rerio*), a small tropical freshwater fish, has received considerable attention as a model for vertebrate embryology and developmental biology (Weinberg, 1992). Although this was the first aquarium species reported to show a tumorigenic response following carcinogen exposure (Stanton, 1965), the potential of this model for cancer research has since gone virtually ignored. We have recently determined this species to be largely resistant to the effects of chemical carcinogens administered in the diet, but more responsive to aquatic exposure protocols, especially at early life stages (Troxel et al., 199x, unpublished). The molecular and genetic bases for development of neoplasias, as well as carcinogen resistance in zebrafish are poorly understood. To begin investigating this, we have recently established the cDNA sequence of a zebrafish *ras* proto-oncogene (Cheng et al., 1996).

Another gene of potential importance to the development of neoplasia in zebrafish is the *p53* tumor suppressor gene. Mutations in *p53* are one of the most common genetic changes found in many forms of human cancer. Loss of negative cellular growth control due to missense, nonsense, and frameshift mutations, which inactivate the wild-type *p53* protein (loss of function), leads to clonal expansion of pre-neoplastic and neoplastic cells. Specific mutant *p53*s have also been shown to transactivate the promoters of certain cellular growth-related genes producing a gain of function which stimulates cell division. It has been speculated that the dual nature of mutant *p53* protein effects, resulting in cellular transformation via independent mechanisms, may explain the preponderance of *p53* mutations observed in human tumors (reviewed in Greenblatt et al., 1994).

Numerous biochemical properties of the wild-type *p53* protein have been described (reviewed in Levine et al., 1991; Cox and Lane, 1995). As noted above, wild-type *p53* suppresses cell division and induces growth arrest at the G1 phase of the cell cycle. In addition, wild-type *p53* binds DNA in a sequence-specific manner to transcriptionally activate certain genes in vivo, including cyclin G, epidermal

growth factor receptor, *MDM2*, and *p21^{waf1-cip1}*; functionally associates with the TATA-binding protein; binds various transcription factors, DNA helicase, and several viral proteins. Expression of wild-type p53 is induced in response to DNA damage. Overexpression of wild-type p53 results in decreased cell growth and, in some cases, apoptosis. Many tumor-associated mutant p53 proteins are known to be defective in normal DNA binding, yet some of these mutant p53s will transactivate promoters of other growth-related genes, such as proliferating cell nuclear antigen, multiple drug resistance/p-glycoprotein and human heat shock protein 70. These multiple functions of the p53 protein are indicative of the central and complex role it occupies in the regulation of metazoan cellular growth and genome integrity.

Complete cDNA sequences for *p53* have been described from human, Rhesus monkey, African green monkey, rat, mouse, golden hamster, cat, cow, sheep, chicken, African clawed frog, rainbow trout, and northern European squid (Matlashewski et al., 1984; Kay et al., 1994; Rigaudy et al., 1989; Soussi et al., 1988b; Oren et al., 1983; Legros et al., 1992; Okuda et al., 1994; Dequiedt et al., 1995b; Dequiedt et al., 1995a; Soussi et al., 1988a; Soussi et al., 1987; Caron de Fromentel et al., 1992; Winge et al., 1995). Although there is sequence diversity among species, p53 amino acid sequences consist of five highly conserved domains specifying critical functions of this protein (reviewed in Soussi et al., 1990; Prives, 1994). In the present study we report the zebrafish *p53* tumor suppressor gene cDNA sequence and describe its temporal expression in freshly fertilized zebrafish embryos.

Results

Isolation of p53-like clones from a zebrafish cDNA library

A PCR-generated, digoxigenin-labeled probe complementary to trout *p53* conserved domain IV was used to screen a zebrafish cDNA library. Approximately 2×10^5 plaques were screened in duplicate by hybridization at room temperature for up to two days. Candidate positive plaques were subjected to second and third screenings to eliminate false positives, and a weakly hybridizing plaque was isolated. The phagemid (pBluescript[®] SK(-), Stratagene) containing this clone, designated Z-p53c, was then excised according to the manufacturer's protocol. Restriction enzyme analysis revealed an insert size of approximately 1.7 kb. This insert was found by sequencing to be 1777 nucleotide pairs in length, and the sequence was then compared with that of the rainbow trout *p53* cDNA (Soussi, 1990). The insert appeared to code for most of a *p53* sequence but lacked sufficient 5' length to contain a region homologous to the consensus of conserved domain I, nor a translation start site.

To evaluate the feasibility of using PCR to screen this library, we used primers (Table 1) specific to the newly identified Z-p53c. PCR with primers Zp53EXN5 and Zp53EX7c generated a strong band of the expected length of about 350 bp. We then employed long PCR protocols (Barnes, 1994) with primer Zp53EX7c against a commercial upstream primer (M13 reverse) specific to the Uni-ZAP[™] XR cloning vector used in constructing the cDNA library. Appropriately sized product bands were excised, purified using the QIAquick system (Qiagen, Chatsworth, CA), ligated and cloned via TA[®] cloning (Invitrogen, San Diego, CA). One candidate clone (Z-p53f) was identified and sequenced. It showed near perfect identity with the previous Z-p53c clone over their coincident lengths, and contained a putative conserved domain I. However, it also failed to show a credible translation start site.

To obtain a full-length clone, the cDNA library was re-screened using a random-primed, radiolabeled probe (Rediprime, Amersham) which was generated from a PCR product from the Z-p53f clone. This screening identified a third clone with more extensive 5' length. The clone, Z-p53m, was also identical to Z-p53c throughout their coincident extent. It possessed an in frame ATG as a potential translation start site, together with sufficient upstream sequence to suggest that further upstream start sites

were unlikely. The complete sequence of the putative zebrafish *p53* cDNA generated from these clones is presented in Figure 1.

Expression of p53, myc, and the murine T homologue in fertilized zebrafish embryos

Northern blot hybridization analysis was carried out on total RNA isolated from synchronously-developing zebrafish embryos (maintained at 28°C) <1, 2, 3, 6, 9, 12, 24, and 48 hours post-fertilization. A single 2.3 kb transcript was identified using the Z-p53c cDNA insert as a probe (Figure 2). The Z-p53-related transcript was most abundant in zygotes and early cleavage-stage embryos (<1 hour post-fertilization) and, thereafter, gradually declined to barely detectable levels at 48 hours. The Z-p53-related mRNA detected in samples taken soon after fertilization is most likely maternally-derived, since activation of zygotic transcription is a characteristic of the midblastula transition (Kimmel, 1989), the onset of which does not occur until cycle 10 (between 2.5 and 3 hours post-fertilization at 28.5°C) (Kane and Kimmel, 1993).

Shown for comparison in Figure 2 are Northern hybridization analyses of the same RNA samples probed for zebrafish *L-myc*, which is also present in maternally-derived mRNA, and zebrafish *N-myc*, which is not detectable prior to the onset of zygotic transcription. Also shown in Figure 2 is reverse-transcription polymerase chain reaction analysis indicating the temporal expression pattern of the zebrafish homologue of the murine *T* gene (*z-T*). *z-T* is detectable (a very weak signal) 3 hours post-fertilization but not prior to the midblastula transition.

Evidence for p53 mRNA alternative splicing in zebrafish

A comparison of the Z-p53f and Z-p53m sequence data (Figure 3) showed close identity at the deduced amino acid level, with the most notable exception being the insertion of a single amino acid (a serine) at position 19. Subsequent PCR on zebrafish genomic templates using primers specific to the cDNA flanking the inserted triplet (see Experimental Procedures) amplified an approximately 350bp product. This product was TA[®] cloned and partially sequenced, analysis of which revealed that the single inserted serine was coded for by the apparent insertion of AAG at the junction of exon 2 and exon 3. Sequence for the 3' end of the intron between exons 2 and 3 revealed two alternative "AG" splice acceptor sites.

Discussion

Sequence comparisons of zebrafish p53 with trout, human and other p53s

Comparison of the zebrafish p53 deduced protein sequence with that of trout and human (Figure 4) reveals that 63% and 48% of the residues, respectively, are identical. Not surprisingly, the alignment reveals quite striking sequence homology in conserved domains I through V between these three species, as well as across all 14 species compared (Figure 4; recently reviewed in Soussi and May, 1996). The most significant divergence between the zebrafish p53 sequence and other species, including trout, is evident in the amino-terminal region of the protein, which also encompasses conserved domain I. Divergence is again more apparent in the carboxy-terminal region.

Expression of z-p53 and other genes in fertilized embryos

Northern analysis employing the Z-p53c cDNA insert as a probe revealed the presence of a single 2.3 kb transcript in total RNA isolated from early zebrafish embryos. Relative levels of the zebrafish p53-related transcript were highest in newly-fertilized eggs, and gradually declined during the first 48 hours of embryonic development. Expression of the transcript within one hour following fertilization indicates that it is contributed, at least in part, maternally.

Our observation that levels of the p53-related transcript declined during zebrafish embryonal development is consistent with earlier reports on *p53* expression during vertebrate embryogenesis. During embryonic development of chickens and mice, there is a post-transcriptionally-regulated decrease in the steady-state levels of p53 protein and an equal decline in *p53* mRNA (Louis et al., 1988); *in situ* hybridization studies reveal that during mouse embryogenesis, *p53* mRNA expression declines in tissues undergoing terminal differentiation (Schmid et al., 1991). Furthermore, it has been demonstrated that p53 (protein and mRNA) levels decline *in vitro* following induced differentiation of cultured murine erythroleukemia cells (Shen et al., 1983; Khochbin et al., 1988) and F9 embryonal carcinoma cells (Chandrasekaran et al., 1982; Dony et al., 1985). These and other findings have led many researchers to

propose that *p53* may play a regulatory role in cells undergoing maturation and differentiation in the early vertebrate embryo.

Alternative splicing of pre-mRNAs

The production of a variety of mRNA splice variants from single genes is a ubiquitously observed phenomenon in metazoan organisms, and an important post-transcriptional regulatory mechanism for gene expression (reviewed in Breitbart, et al., 1987). Alternative splice patterns generated during pre-mRNA processing can produce different mature transcripts which encode related protein isoforms which may or may not be functional. One alternate splice pattern involves the use of cryptic donor or acceptor sites, which, when in frame, may produce either increased or decreased coding length of the resultant mRNA. Alternately spliced mRNAs arising as the result of splice donor or acceptor site mutations have been detected in numerous tumors and tumor cell lines, indicating that alternative splicing can generate protein variants with transforming potential, including *p53* (Magnusson, et al., 1995), *WT1* (Englert, et al., 1995), and *mdm2* (Haines, et al., 1994). Alternative splicing of the *p53* transcript to produce distinct functional forms of the protein has thus far only been documented in the mouse (Bayle, et al., 1995; Wu, et al., 1994, 1995).

In this work we present initial evidence for possible alternative splicing in the zebrafish *p53* pre-mRNA transcript. The sequence data resulting from clones Z-p53f and Z-p53m and the genomic PCR product reveals that the inserted triplet AAG in Z-p53f is positioned precisely at the intron-exon boundary of exon 3 (Figure 3). The Z-p53f clone represents an mRNA that could result from use of the "AG" immediately upstream of the inserted triplet as the splice acceptor site. Use of this AG would then prevent the subsequent AAG from being incorporated into the excised intron and, thus, would result in its insertion into the mature message. Alternatively, clone Z-p53f may have resulted from the presence of a *p53* polymorphism or gene duplication in this species. Additional studies are needed to distinguish these hypotheses and to assess their functional significance.

Experimental Procedures

DNA isolation from whole zebrafish

Whole adult zebrafish were cut into small pieces and frozen immediately in liquid nitrogen. For DNA isolation, 0.1g of pulverized tissue was added per 1.2ml of lysis buffer [0.1M NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS, and 0.1mg/ml of proteinase K (Amresco, Solon, OH)] and incubated overnight at 37°C. The DNA was extracted once each with phenol/chloroform and chloroform, and precipitated with 2.5 volumes of 95% ethanol at a final NaCl concentration of 0.2M. The DNA precipitates were dissolved in TE buffer and treated with RNAase (Sambrook et al., 1989).

Primers

All primers (Table 1) were synthesized by the Central Services Laboratory of the Center for Gene Research and Biotechnology, Oregon State University. Primers were dissolved in water or TE and used without further purification.

Non-radiolabeled probe preparation by PCR amplification

A non-radiolabeled probe, based on conserved region IV of the rainbow trout *p53* gene, was amplified via polymerase chain reaction (PCR) by p53 E7 and cE7 primers, using the rainbow trout *p53* cDNA clone as template for the incorporation of digoxigenin-11-dUTP. PCR was accomplished with Replitherm™ polymerase (Epicentre Technologies, Madison, WI) under the following conditions: one initial cycle of 95°C denaturation for 6 min; followed by 2 cycles consisting of 95°C denaturation for 2 min, 60°C annealing for 45 s, and 74°C extension for 25 s; and lastly, 40 cycles consisting of 95°C denaturation for 30 s, 60°C annealing for 45 s, and 74°C extension for 25 s. The PCR products were purified through a Microcon-30™ membrane concentrator (Amicon, Beverly, MA) to remove excess primers and dNTPs. Before use, the probe purity and quantity were assessed by electrophoresis against known standards on polyacrylamide with ethidium stain and UV fluorescence.

cDNA library plaque screening via non-radiolabeled probing

The plaque-lift hybridization methods for cDNA library screening are described elsewhere (Sambrook et al., 1989). The non-radiolabeled screening and detection system was from Boehringer Mannheim (Indianapolis, IN); their protocols were used without modification. Candidate positive spots were subjected to second and third screenings by the same procedures. The phagemid from a single confirmed positive plaque was then isolated.

Preparation of radiolabeled probe

The ^{32}P -labeled probe was prepared using an Amersham (Arlington Heights, IL) "rediprime" random primer labeling kit, as per the manufacturer's instructions. Template DNA was a PCR product generated by using the primers Zp53CU and cZ53-137 with the Z-p53f clone. The product was purified over a Qiagen (Chatsworth, CA) QIAquick PCR purification column following the manufacturer's protocol. The resulting radiolabeled probe was used according to the plaque hybridization protocol supplied by Stratagene (La Jolla, CA) with the Zap-cDNATM Synthesis Kit.

DNA sequencing

The plasmid sequencing of isolated clones was carried out by Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH) as per the manufacturer's protocols, using [$\alpha^{35}\text{S}$]-dATP (Dupont NEN, Boston, MA). All other DNA sequencing was either performed in-house using ΔTaq Version 2.0 (United States Biochemical Corp.) or at the Oregon State University Center for Gene Research and Biotechnology Central Services Laboratory, using PE Applied Biosystems, Inc., automated fluorescent sequencing.

Probes for Northern blot hybridization analyses

The 1.8 kb insert of clone Z-p53c from the library was isolated from the pBluescript[®] phagemid following *EcoRI/XhoI* digestion. Zebrafish *myc* probes consisted of a 560-bp *XmnI/EcoRI* fragment specific for zN-*myc* and a 1.4 kb *EcoRI* fragment specific for zL-*myc*, as described (Schreiber-Agus et al., 1993). Digests were electrophoresed on a 1% agarose gel; inserts were visualized by ethidium bromide fluorescence,

excised from the gel, and recovered using a Geneclean™ II kit (BIO 101, La Jolla, CA) according to the manufacturer's instructions. [$\alpha^{32}\text{P}$]-dNTP-labeled probes were prepared by random-primed labeling.

Preparation and analysis of embryo RNA

Total RNA was isolated from wild-type zebrafish embryos (maintained at 28°C) at various times post-fertilization according to the guanidinium method (Kingston et al., 1990). Samples of total RNA (18 µg/lane) were fractionated by electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes. Hybridizations were carried out for 17 hr at 42°C in the presence of 50% formamide, 5X Denhardt's, 5X SSC, 0.1% SDS, and 250 mg/ml sonicated calf thymus DNA.

RT-PCR for the zebrafish homologue of the murine T gene

RT-PCR was carried out using a commercial reverse transcription system (Promega) according to the manufacturer's instructions. Total RNA (1 µg) was prepared in a 20 µl reaction mixture containing 5 mM MgCl₂, 1X RT buffer, 1 mM dNTPs, 1 U/ml ribonuclease inhibitor, 16 U AMV reverse transcriptase, and 0.5 µg oligo(dT)₁₅ primer. After incubation at 42°C for 30 minutes, 5 µl of this mixture were amplified in a total volume of 100 µl containing 1.75 mM MgCl₂, 1X PCR buffer, 0.15 µM dNTPs, 2.5 U Taq polymerase (Perkin-Elmer), and 0.6 µM oligonucleotide primers. Primer sequences and amplification conditions were described previously (Schulte-Merker et al., 1992).

PCR screening of the cDNA library

M13 reverse, an upstream primer specific to the Uni-Zap SK⁻ phagemid vector, was used against Zp53EX7c (Table 1). PCR was conducted using an Expand™ High Fidelity reagent kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) in a Stratagene Robocycler 96 with a gradient annealing block. PCR conditions were modified from the Boehringer Mannheim protocol in the following manner. Instead of incrementing extension times with each cycle, the increments were combined into three stages of 12 cycles each. Denaturation to 95°C was accomplished in very short times (Barnes, 1994) by using a 99°C block temperature for 14 s. Cycle parameters were: an initial hold at 99°C for 12 s to bring the reactions from 4

°C preparation temperature, 11 cycles consisting of 99 °C denaturation for 14 s, 49 to 56 °C annealing for 1 min in the Robocycler gradient block, and 70 °C extension for 2 min; then 11 more cycles consisting of 99 °C denaturation for 14 s, 49 to 56 °C annealing for 1 min, and 70 °C extension for 3 min 30 s; then 11 more cycles consisting of 99 °C denaturation for 14 s, 49 to 56 °C annealing for 1 min, and 70 °C extension for 5 min; followed by a final extension of 75 °C for 10 min. All reagents and other parameters are as specified in the Boehringer-Mannheim protocol.

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Legends to Figures

Figure 1. The cDNA and deduced amino acid sequences of zebrafish *p53*. Sequence read from clone Z-p53c includes bases 341 (emboldened A^c) through 2105; sequence read from clone Z-p53m includes bases 1 through 1110 (emboldened C^s). PCR and sequencing primers are underlined and identified with superscripts corresponding to their annotation in Table 1. The sequence has been submitted to GenBank under the accession number U60804.

Figure 2. Temporal expression pattern of *z-p53*-related mRNA during early zebrafish development, compared to *zL-myc*, *zN-myc*, and the zebrafish homologue of the murine *T* gene (*z-T*). Analyses were carried out as described in the text. The top three panels show results obtained in Northern blot hybridization analyses using radio-labelled *z-p53* cDNA, *zL-myc* subclone cDNA, and *zN-myc* subclone cDNA, respectively, as probes (RNA from embryos 2 hours post-fertilization was available in limiting quantity and was only electrophoresed on the *z-p53* gel). The bottom panel shows expression of *z-T*, as revealed by RT-PCR (RNA from embryos <1 hour post-fertilization was unavailable; the first lane in this panel shows absence of amplification observed in a control sample not containing RNA).

Figure 3. Partial nucleotide sequence data for clones Z-p53m and Z-p53f, and the intron sequence between exons 2 and 3 showing two alternate splice sites. Z-p53m is the top line of each pair, Z-p53f is the bottom line of each pair. The deduced amino acid sequence is shown as the one-letter code; amino acid differences are in parentheses. The inserted amino acid at position 19 of Z-p53f is bracketed and its encoding triplet is underlined. The emboldened "T" is actually the first nucleotide in exon 3. Shaded boxes outline the alternative splice acceptor sites at the 3' junction of the intron. Use of the upstream "AG" would result in the addition of 3 nucleotides (AAG) to the 5' end of exon 3.

Figure 4. Comparison of the predicted p53 amino acid sequences for those species for which complete coding sequences are currently available. Multiple alignment relative to the human sequence was performed using the Clustal W program (Thompson, et al., 1994), with minor adjustments made by hand to optimize the homology. Conserved domains I through V are depicted by shaded boxes. Gaps inserted in the sequences to obtain an alignment are indicated by dashes. The specific sequences used for this comparison include human (Matlashewski et al., 1984), Rhesus monkey (Kay et al., 1994), African green monkey (Rigaudy et al., 1989), rat (Soussi et al., 1988b), mouse (Oren et al., 1983), golden hamster (Legros et al., 1992), cat (Okuda et al., 1994), cow (Dequiedt et al., 1995b), sheep (Dequiedt et al., 1995a), chicken (Soussi et al., 1988a), African clawed frog (Soussi et al., 1987), rainbow trout (Caron de Fromentel et al., 1992), and northern European squid (Winge et al., 1995). Not shown in the squid sequence are an additional 137 amino acids extending beyond the 3' glutamine.

GTTTAGTGGAGAGGAGGTGCGCAAAATCAATTCTTGCAAAGCA																			43	
ATG	GCG	CAA	AAC	GAC	AGC	CAA	GAG	TTC	GCG	GAG	CTC	TGG	GAG	AAG	AAT	TTG	ATT	ATT	CAG	103 ^a
M	A	Q	N	D	S	Q	E	F	A	E	L	W	E	K	N	L	I	I	Q	
CCC	CCA	GGT	GGT	GGC	TCT	TGC	TGG	GAC	ATC	ATT	AAT	GAT	GAG	GAG	TAC	TTG	CCG	GGA	TCG	163
P	P	G	G	G	S	C	W	D	I	I	N	D	E	E	Y	L	P	G	S	
TTT	GAC	CCC	AAT	TTT	TTT	GAA	AAT	GTG	CTT	GAA	GAA	CAG	CCT	CAG	CCA	TCC	ACT	CTC	CCA	223
F	D	P	N	F	F	E	N	V	L	E	E	Q	P	Q	P	S	T	L	P	
CCA	ACA	TCC	ACT	GTT	CCG	GAG	ACA	AGC	GAC	TAT	CCC	GGC	GAT	CAT	GGA	TTT	AGG	CTC	AGG	283 ^b
P	T	S	T	V	P	E	T	S	D	Y	P	G	D	H	G	F	R	L	R	
TTC	CCG	CAG	TCT	GGC	ACA	GCA	AAA	TCT	GTA	ACT	TGC	ACT	TAT	TCA	CCG	GAC	CTG	AAT	AAA	343 ^c
F	P	Q	S	G	T	A	K	S	V	T	C	T	Y	S	P	D	L	N	K	
CTC	TTC	TGT	CAG	CTG	GCA	AAA	ACT	TGC	CCC	GTT	CAA	ATG	GTG	GTG	GAC	GTT	GCC	CCT	CCA	403 ^d
L	F	C	Q	L	A	K	T	C	P	V	Q	M	V	V	D	V	A	P	P	
CAG	GGC	TCC	GTG	GTT	CGA	GCC	ACT	GCC	ATC	TAT	AAG	AAG	TCC	GAG	CAT	GTG	GCT	GAA	GTG	463 ^e
Q	G	S	V	V	R	A	T	A	I	Y	K	K	S	E	H	V	C	E	V	
GTC	CGC	AGA	TGC	CCC	CAT	CAT	GAG	CGA	ACC	CCG	GAT	GGA	GAT	AAC	TTG	VCG	CCT	GCT	GGT	523
V	R	R	C	P	H	H	E	R	T	P	D	G	D	N	L	A	P	A	G	
CAT	TTG	ATA	AGA	GTG	GAG	GGC	AAT	CAG	CGA	GCA	AAT	TAC	AGG	GAA	GAT	AAC	ATC	ACT	TTA	583
H	L	I	R	V	E	G	N	Q	R	A	N	Y	R	E	D	N	I	T	L	
AGG	CAT	AGT	GTT	TTT	GTC	CCA	TAT	GAA	GCA	CCA	CAG	CTT	GGT	GCT	GAA	TGG	ACA	ACT	GTG	643
R	H	S	V	F	V	P	Y	E	A	P	Q	L	G	A	E	W	T	T	V	
CTA	CTA	AAC	TAC	ATG	TGC	AAT	AGC	AGC	TGC	ATG	GGG	GGG	ATG	AAC	CGC	AGG	CCC	ATC	CTC	703 ^f
L	L	N	Y	M	C	N	S	S	C	M	G	G	M	N	R	R	P	I	L	
ACA	ATC	ATC	ACT	CTG	GAG	ACT	CAG	GAA	GGT	CAG	TTG	CTG	GGC	CGG	AGG	TCT	TTT	GAG	GTG	763
T	I	I	T	L	E	T	Q	E	G	Q	L	L	G	R	R	S	F	E	V	
CGT	GTG	TGT	GCA	TGT	CCA	GGC	AGA	GAC	AGG	AAA	ACT	GAG	GAG	AGC	AAC	TTC	AAG	AAA	GAC	823
R	V	C	A	C	P	G	R	D	R	K	T	E	E	S	N	F	K	K	D	
CAA	GAG	ACC	AAA	ACC	ATG	GCC	AAA	ACC	ACC	ACT	GGG	ACC	AAA	CGT	AGT	TTG	GTG	AAA	GAA	883
Q	E	T	K	T	M	A	K	T	T	T	G	T	K	R	S	L	V	K	E	
TCT	TCT	TCA	GCT	ACA	TTA	CGA	CCT	GAG	GGG	AGC	AAA	AAG	GCC	AAG	GGC	TCC	AGC	AGC	GAT	943
S	S	S	A	T	L	R	P	E	G	S	K	K	A	K	G	S	S	S	D	
GAG	GAG	ATC	TTT	ACC	CTG	CAG	GTG	AGG	GGC	AGG	GAG	CGT	TAT	GAA	ATT	TTA	AAG	AAA	TTG	10

Figure 2

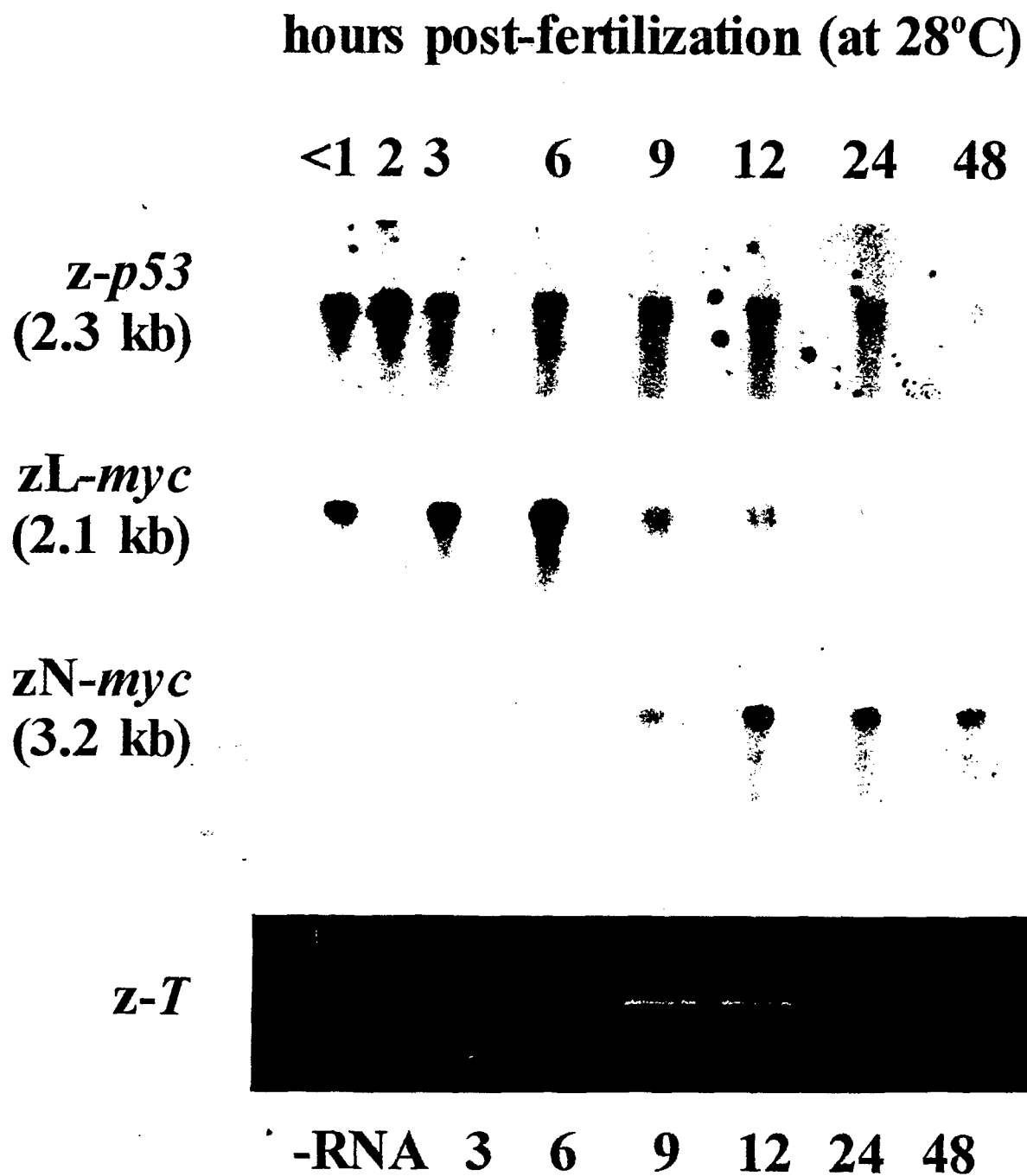


Figure 3

	ATG GCG CAA AAC GAC AGC CAA GAG TTC GCG GAG CTC TGG GAG AAG AAT TTG AT	T
	M A Q N D S Q E F A E L W E K N L I	
EXON 2	(D)	[S]
	G CAA AAC GAC AGC CAA GAG TTC GCG GAC CTC TGG GAG AAG AAT TTG AT	<u>A AG T</u>
	AAGGGTCGCACTCCTGATACACAACGATCCTCTTTTTCCTGCCTCTCTAAATTTCCCGTATTTTGTAGCAGCAGC	
	CATGTCAGGTTGCTATAATGTACCTGTCTTAATATTTTGGTGTTGCTTGTTAAGATGCATCTGTCGAACGTATT	
	GTTTATCTGGAGTTCTTTGTATGAGCTTCAACAGTTAATACTAATTTCTCTCTCTCCTTTCAATTGTCTC	<u>AGAAG</u>
		INTRON
	ATT CAG CCC CCA GGT GGT GGC TCT TGC TGG GAC ATC ATT AAT GAT GAG	
	I Q P P G G G S C W D I I N D E	
	ATT CAG CCC CCA GGA GGT GGC TCT TGC TGG GAC ATC ATT AAT GAT GAG	EXON 3

Figure 4

ZEBRAFISH	-----MAQ-----	NDSQE-FAELWEKNL	---IIQPP---GGGS	CWDIINDEEYLPGSF	DPNFFENVLEE----	-----Q	53
TROUT	-----MADLAENVSL	PLSQESFEDLWKMNL	NLVAVQPPET-ESWV	GYDNFMMEAPLQVEF	DPSLFEVSATEP----	-----A	67
SQUID	-----MSQG-TS---	PNSQETFNLLWDSLE	QVTANEYTOIHERGV	GYEYHEAEPDQTSLE	ISAYRIAQPDYGRS	ESYDLLNPIINQIPA	81
FROG	-----MEPSSETGMDP	PLSQETFFDLWLLP	-----LSSSV-----	-DPLQTVTCRLDNLS	EFF--DYPLAADMT	-----V	53
CHICKEN	-----MAEMEPL	LEPTVFMDLWMLP	-----YS	MQQLPLPEDHSNWQE	LSPLEPSDPPPPPP	P-----PLPLAAAA	64
COW	---MEESQAEINVEP	PLSQETFFDLWLLP	ENNLLSSEL--SAPV	DDLLPY-TDVATWLD	ECP--NEAPQMPEPS	-----APAA	71
SHEEP	---MEESQAEIGVEP	PLSQETFFDLWLLP	ENNLLSSEL--SAPV	DDLLPYSEDVVTWLD	ECP--NEAPQMPEP	-----	67
CAT	---MQEPPLELTIEP	PLSQETFFSELWLLP	ENNVLSEL--SSAM	NELPLS-EDVANWLD	EAP--DDASGMSAVP	-----APAA	71
HAMSTER	---MEEPQSDLSIEL	PLSQETFFDLWLLP	PNNVLSLTP--SSDS	IEELFLSENVAWGLE	DPG---EALQGSAAA	AAPAAPAAEDPVAET	82
MOUSE	MTAMEESQSDISLEL	PLSQETFFSGLWLLP	PEDILP-----SPHC	MDDLPLPDQVEEFEE	GPS---EALRVSG--	-----APAAQDPVTET	76
RAT	---MEDSQSDMSIEL	PLSQETFFSGLWLLP	PDDILPTTATGSPNS	MEDLFLPDQVAELLE	GPE---EALQVS--	-----APAAQEPGTEA	77
GR MONKEY	---MEEPQSDPSIEP	PLSQETFFDLWLLP	ENNVLSPLP--SQAV	DDLMLSPDDLAQWLT	EDPGPDEAPRMSEAA	P-----HMAPTPAA	79
RH MONKEY	---MEEPQSDPSIEP	PLSQETFFDLWLLP	ENNVLSPLP--SQAV	DDLMLSPDDLAQWLT	EDPGPDEAPRMSEAA	P-----PMAPTPAA	79
HUMAN	---MEEPQSDPSVEP	PLSQETFFDLWLLP	ENNVLSPLP--SQAM	DDLMLSPDDIEQWFT	EDPGPDEAPRMPEAA	P-----PVAPAPAA	79

I

ZEBRAFISH	PQP-----	-----STLP---	-----PTSTVPETS	DYPGDHGFRLRFPQ-	SGTAKSVTCTYSPDL	NKFLCQLAKTCFVQM	113
TROUT	PQPSIST-----	-----LDTGSP---	-----PTSTVPTTS	DYPGALGFQRLRFLQ-	SSTAKSVTCTYSPDL	NKFLCQLAKTCFVQI	133
SQUID	PMPIADTQNNPLVNH	CPYEDMPVSSTPYSP	HDHVQSPQPSVPSNI	KYPGEYVFEMSAQF	SKETKSTTWTYSEKL	DKLYVRMATTCFVRF	171
FROG	LQEGLMG-----	-----NAVPTV---	-----TSCAVPSTE	DYAGKYGLQLDFQO-	NGTAKSVTCTYSPDL	NKFLCQLAKTCFLLV	119
CHICKEN	PPPLNP-----	-----TPPRAA---	-----PSPVVPSTE	DYGGDFDFRVGFEV-	AGTAKSVTCTYSPVL	NKLYCRLAKTCFVQV	130
COW	PPPATPA-----	-----PATSWP---	-----LSSFVPSQK	TYPGNYGFRGLFGLQ-	SGTAKSVTCTYSPSL	NKFLCQLAKTCFVQL	137
SHEEP	PAQAALA-----	-----PATSWP---	-----LSSFVPSQK	TYPGNYGFRGLFGLH-	SGTAKSVTCTYSPSL	NKFLCQLAKTCFVQL	133
CAT	PAPATPA-----	-----PAISWP---	-----LSSFVPSQK	TYPGAYGFHLGFLQ-	SGTAKSVTCTYSPPL	NKFLCQLAKTCFVQL	137
HAMSTER	PAPVASA-----	-----PATPWP---	-----LSSSVPSYK	TYQGNYGFRGLFGLH-	SGTAKSVTCTYSPVL	NKFLCQLAKTCFVQL	148
MOUSE	PGPVAPA-----	-----PATPWP---	-----LSSFVPSQK	TYQGNYGFRGLFGLQ-	SGTAKSVTCTYSPPL	NKFLCQLVKTCTFVQL	142
RAT	PAPVAPA-----	-----SATPWP---	-----LSSSVPSQK	TYQGNYGFRGLFGLQ-	SGTAKSVTCTYSPSL	NKFLCQLAKTCFVQL	143
GR MONKEY	PTPAAPA-----	-----PAPSWP---	-----LSSSVPSQK	TYHGSYGFRGLFGLH-	SGTAKSVTCTYSPDL	NKMFQQLAKTCFVQL	145
RH MONKEY	PTPAAPA-----	-----PAPSWP---	-----LSSSVPSQK	TYHGSYGFRGLFGLH-	SGTAKSVTCTYSPDL	NKMFQQLAKTCFVQL	145
HUMAN	PTPAAPA-----	-----PAPSWP---	-----LSSSVPSQK	TYQGSYGFRGLFGLH-	SGTAKSVTCTYSPAL	NKMFQQLAKTCFVQL	145

II

ZEBRAFISH	VVDVAPPQGSVVRAT	AIYKKSEHVAEVR	CPHHERTPDGDN-LA	PAGHLIRVEGNQRAN	YREDNITLRHSVFPV	YEAPQLGAEWTTVL	202
TROUT	VVDHPPPPGAVVRAL	AIYKKLSDVADVVR	CPHHQSTSENNEGPA	PRGHLVRVEGNQRSE	YMEDGNTLRHSVLPV	YEPQVGSSECTTVL	223
SQUID	KTARPPPSGCQIRAM	PIYMKPEHVQEVVCR	CPNHATAKEHNEKHP	APLHVRCHEKL-AK	YHEDKYSGRQSVLIP	HEMPQAGSEWVNL	260
FROG	RVESPPPPGSLILRAT	AVYKKSEHVAEVR	CPHHERSVEPEGEDAA	PPSHLMRVEGNLQAY	YMEDVNSGRHSVCVP	YEGPQVGTCTTVL	209
CHICKEN	RVGVAPPPGSLRAV	AVYKKSEHVAEVR	CPHHERC GGTDGLA	PAQHLIRVEGNPQAR	YHDDETTKRHSVVVP	YEPPEVGSCTCTTVL	220
COW	WVDSPPPPGTRVRAM	AIYKKLEHMTVEVR	CPHHERSDYS DGLA	PPQHLIRVEGNLRAE	YLDNRNTFRHSVVVP	YESPEIDSECTTIH	227
SHEEP	WVDSPPPPGTRVRAM	AIYKKLEHMTVEVR	SPHHERSDYS DGLA	PPQHLIRVEGNLRAE	YFDDRNTFRHSVVVP	YESPEIESECTTIH	223
CAT	WVRSPPPPGTCVRAM	AIYKKSEFMTVEVR	CPHHERCPSDSDGLA	PPQHLIRVEGNLHAK	YLDNRNTFRHSVVVP	YEPPEVGSCTCTTIH	227
HAMSTER	WVSTPPPPGTRVRAM	AIYKKLQYMTVEVR	CPHHERSSE-GDGLA	PPQHLIRVEGNMHA	YLDNRNTFRHSVVVP	YEPPEVGSCTCTTIH	237
MOUSE	WVSATPPAGSRVRAM	AIYKKSQHMTVEVR	CPHHERCSD-GDGLA	PPQHLIRVEGNLYPE	YLEDQRTFRHSVVVP	YEPPEAGSEYTTIH	231
RAT	WVTSTPPPGTRVRAM	AIYKKSQHMTVEVR	CPHHERCSD-GDGLA	PPQHLIRVEGNPYAE	YLDNRNTFRHSVVVP	YEPPEVGSCTCTTIH	232
GR MONKEY	WVDSTPPPGSRVRAM	AIYKQSQHMTVEVR	CPHHERCSD-SDGLA	PPQHLIRVEGNLRAE	YSDNRNTFRHSVVVP	YEPPEVGSCTCTTIH	234
RH MONKEY	WVDSTPPPGSRVRAM	AIYKQSQHMTVEVR	CPHHERCSD-SDGLA	PPQHLIRVEGNLRAE	YSDNRNTFRHSVVVP	YEPPEVGSCTCTTIH	234
HUMAN	WVDSTPPPGTRVRAM	AIYKQSQHMTVEVR	CPHHERCSD-SDGLA	PPQHLIRVEGNLRAE	YLDNRNTFRHSVVVP	YEPPEVGSCTCTTIH	234

III

ZEBRAFISH	LYNMCNSSCMGMNRR	PILTIITLETQEQGL	LGRNSFEVRVCACPG	RDRKTEESNFKKDQ-	-ETK-TMAKTTGTGK	RSLVKESSSATLRPE	289
TROUT	YNFMCNSSCMGMNRR	PILTIITLETQEQGL	LGRNSFEVRVCACPG	RDRKTEEINLKKQEE	TTLE-TKTKPAQGIK	RAMKEASLPAPQ-PG	311
SQUID	YQFMCILGSCVGPNNR	PIQLVFTLE-KDNQV	LGRRAVEVRICACPG	RDRKADEKASLVSKP	-----PSP-KKNGFPQ	RSVLVNTDITKIT--	342
FROG	YNFMCNSSCMGMNRR	PILTIITLETQGLL	LGRRCFEVRVCACPG	RDRRTEEDNYTKRG	-----LKPSPGKR	ELAHPPSPSEPPL---	288
CHICKEN	YNFMCNSSCMGMNRR	PILTIITLETGPGGL	LGRRCFEVRVCACPG	RDRKIEEENFRKRG	-----AGGVAK	RAMSEPTAEAPL---	298
COW	YNFMCNSSCMGMNRR	PILTIITLETSCGNL	LGRNSFEVRVCACPG	RDRRTEENLRKKGQ	-----SCPEPPPRSTK	RALPTNTSSSPQ---	310
SHEEP	YNFMCNSSCMGMNRR	PILTIITLETDSRGNL	LGRSSFEVRVCACPG	RDRRTEENFRKKGQ	-----SCPEPPPGSTK	RALPSSTSSSPQ---	306
CAT	YNFMCNSSCMGMNRR	PILTIITLETDSNGKL	LGRNSFEVRVCACPG	RDRRTEENFRKKGQ	-----PCPEPPPGSTK	RALPSTSSSTPP---	310
HAMSTER	YNFMCNSSCMGMNRR	PILTIITLETDPGSLN	LGRNSFEVRVCACPG	RDRRTEENFRKKGQ	-----PCPELPKPSAK	RALPTNTSSSPQ---	320
MOUSE	YKYM CNSSCMGMNRR	PILTIITLETDSGNL	LGRDSFEVRVCACPG	RDRRTEENFRKKEV	-----LCPELPKPSAK	RALPTCTSSSPQ---	314
RAT	YKYM CNSSCMGMNRR	PILTIITLETDSGNL	LGRDSFEVRVCACPG	RDRRTEENFRKKEE	-----HCPELPKPSAK	RALPTSTSSSPQ---	315
GR MONKEY	YNFMCNSSCMGMNRR	PILTIITLETDSGNL	LGRNSFEVRVCACPG	RDRRTEENFRKKGQ	-----PCHELPKPSAK	RALPNTSSSPQ---	317
RH MONKEY	YNFMCNSSCMGMNRR	PILTIITLETDSGNL	LGRNSFEVRVCACPG	RDRRTEENFRKKGQ	-----PCHQLPPGSTK	RALPNTSSSPQ---	317
HUMAN	YNFMCNSSCMGMNRR	PILTIITLETDSGNL	LGRNSFEVHVCACPG	RDRRTEENLRKKGQ	-----PHHELPPGSTK	RALPNTSSSPQ---	317

IV

V

ZEBRAFISH	GSKKAKGS--SSDEE	IFTLQVRGRERYEIL	KKLNDLSLELSDVPA	SDAEKYRQKFMKTKN	KENRESSEPKQGKKL	MVKD---EGRSDSD-	373
TROUT	ASKKTKSSPAVSDDE	IYTLQIRGKEKYEML	KKFNDSLELSELVVP	ADADKYRQKCLTKRV	AKRDFGVGPKKRKKL	LVKE---E-KSDSD-	396
SQUID	PKKRKIDD-----E	CFTLKVGRGRERYEIL	CKLRDIMEAARIPE	AERLLYKQERQAPIG	RLTSLPSSSSNGSQD	GSRSSTAFSTSDSSQ	426
FROG	PKKRLVVVD--DDEE	IFTLRIKGRSRYEMI	KKLNDALQLQESLDQ	QKVTIKCRKCRDEIK	-----P--KKGKKL	LVKD---E-QPDSE-	363
CHICKEN	PKKRVLMPD--N--E	IFYLQVRGRERYEIL	KEINEALQLAEG--	GSAPRPSKGRVYKVE	-----GPQSPCKKKL	LQK-----G--SD-	367
COW	PKKKPLDG-----E	YFTLQIRGFRKRYEMF	RELNDALQLKDALD-	GREPGESRAHSSHLK	SKK--RPSPSCHKKP	MLKR---E-GPDS-	386
SHEEP	QKKKPLDG-----E	YFTLQIRGRKRFEMF	RELNEALELMDAQA-	GREPGESRAHSSHLK	SKK--GPSPSCHKKP	MLKR---E-GPDS-	382
CAT	QKKKPLDG-----E	YFTLQIRGRERFEMF	RELNEALELMDAQA-	GKEPGESRAHSSHLK	AKK--QGSTSRHKKP	MLKR---E-GLSD-	386
HAMSTER	PKRKTLDG-----E	YFTLQIRGRERFEMF	RELNEALELMDAQA-	LKASEDSGAHSSYLK	SKK--GQSASRLKKL	MIKR---E-GPDS-	396
MOUSE	QKKKPLDG-----E	YFTLQIRGRKRFEMF	RELNEALELMDAQA-	TEESGDSRAHSSYLK	TKK--QGSTSRHKKP	MVKK---V-GPDS-	390
RAT	QKKKPLDG-----E	YFTLQIRGRERFEMF	RELNEALELMDAQA-	AEESGDSRAHSSYPK	TKK--QGSTSRHKKP	MIKK---V-GPDS-	391
GR MONKEY	PKKKPLDG-----E	YFTLQIRGRERFEMF	RELNEALELMDAQA-	GKEPAGSRAHSSHLK	SKK--QGSTSRHKKF	MFKT---E-GPDS-	393
RH MONKEY	PKKKPLDG-----E	YFTLQIRGRERFEMF	RELNEALELMDAQA-	GKEPAGSRAHSSHLK	SKK--QGSTSRHKKF	MFKT---E-GPDS-	393
HUMAN	PKKKPLDG-----E	YFTLQIRGRERFEMF	RELNEALELMDAQA-	GKEPAGSRAHSSHLK	SKK--QGSTSRHKKL	MFKT---E-GPDS-	393

Table 1: PCR Primers

Trout specific primers:

p53 E7	5'- GGG ATC AGA GTG TAC CAC TG - 3'	5' end of exon 7
p53 cE7	5'- GTG TCT CCA GGG TGA TGA TG - 3'	3' end of exon 7

Zebrafish specific primers:

^c Z53-137	5'- GGA CCA CTT CAG CCA CAT GC - 3'
^d Zp53EXN5	5'- AAA CTC TTC TGT CAG CTG GC - 3'
^f Zp53EX7c	5'- GTG ATG ATT GTG AGG ATG GG - 3'
^b Zp53BD	5'- TCG CTT GTC TCC GGA ACA GTG G -3'
^a Zp53CU	5'- GCC AAG AGT TCG CGG ACC TCT - 3'

Vector Primer:

M13 rev	5'- GGA AAC AGC TAT GAC CAT G - 3'
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Regulation of Cytochrome P450 Expression in a Novel Liver Cell Line from Zebrafish (*Brachydanio rerio*)

C. L. Miranda,*† Paul Collodi,‡¹ Xine Zhao,* David W. Barnes,‡ and Donald R. Buhler*†²

*Department of Agricultural Chemistry, †Marine/Freshwater Biomedical Sciences Center, and ‡Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-7307

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The expression and induction of cytochrome P450 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and β -naphthoflavone (BNF) in a new liver cell line from adult zebrafish (*Brachydanio rerio*) were studied. Subcellular fractions from control, BNF- or TCDD-treated cells did not show detectable bands in immunoblots probed with antibodies to the constitutive forms of trout P450 (LMC1, LMC2, LMC3, LMC4, and LMC5), suggesting that either zebrafish liver cells lack P450s closely related to those constitutively expressed in trout or that the concentrations of the orthologous P450s were too low to be detected. However, upon exposure to TCDD, the cells expressed a major immunoreactive 54-kDa protein and a minor 50-kDa protein recognized by antibodies to rainbow trout P4501A1. These immunoreactive proteins were observed in microsomal and mitochondrial fractions of TCDD-treated cells but were not detected in cell cultures treated with dimethyl sulfoxide (DMSO) (vehicle control) or BNF. The activities of ethoxyresorufin *O*-deethylase (EROD) and 7,12-dimethylbenzanthracene (DMBA) hydroxylase were markedly increased by TCDD but not by BNF in this cell line. EROD activity was more sensitive than DMBA hydroxylase activity of TCDD-treated liver cells to diagnostic inhibitors such as α -naphthoflavone and anti-trout P4501A1 IgG. The TCDD-treated cells converted DMBA to various metabolites, one of which is the putative proximate carcinogen, DMBA-3,4-diol. These results suggest that TCDD, but not BNF, induces one or possibly two forms of P450 immunologically and functionally related to trout P4501A1, in cultured zebrafish liver cells. © 1993 Academic Press, Inc.

Cells in primary culture and established cell lines provide an attractive model for the study of the expression

and regulation of cytochromes P450 *in vitro* as one can easily examine the various factors that influence expression. However, there are limitations in utilizing cell culture for P450 studies. For example, primary hepatocyte cultures of animal or human origin rapidly lose some species of P450 (1-5). In some cases, P450s that are induced *in vivo* are not induced *in vitro* (1). Nonetheless, cultured cells remain as valuable models for understanding mechanisms of P450 induction. Cell lines, such as those derived from mouse liver, respond to inducers of different forms of P450 belonging to the 2B, 1A, 2E, and 3A subfamilies (6), making them potentially useful for elucidating the mechanisms by which certain xenobiotics induce P450.

Studies with P450 induction with fish cells in culture have focused on P4501A1 (7-11). The polycyclic aromatic hydrocarbons (PAH)³ 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and β -naphthoflavone (BNF) were found to be potent inducers of P4501A1 in primary rainbow trout hepatocyte cultures (7, 8). TCDD also is an inducer of aryl hydrocarbon hydroxylase (AHH) activity in the rainbow trout hepatoma cell line RTH-149, which contains the Ah receptor (9). TCDD likewise induces ethoxyresorufin *O*-deethylase (EROD) activity in zebrafish haploid embryo-derived cells (10), diploid embryo-derived cells and in primary cultures of zebrafish hepatocytes (11).

A new cell line derived from the liver of adult zebrafish has been established and passaged several times in one of our laboratories (10). These cells are potentially useful for studying many differentiated liver functions including induction of cytochrome P450 and they may be able to serve as *in vitro* alternatives to animal experimentation

³ Abbreviations used: PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BNF, β -naphthoflavone; AHH, aryl hydrocarbon hydroxylase; EROD, ethoxyresorufin *O*-deethylase; [³H]DMBA, dimethyl[G-³H]benz[a]anthracene; PBS, phosphate-buffered saline; EGF, epidermal growth factor; ZF-L, zebrafish liver; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; ANF, α -naphthoflavone; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.

¹ Current address: Department of Animal Sciences, Purdue University, West Lafayette, IN 47907.

² To whom correspondence should be addressed.

for toxicological and carcinogenesis studies. The present work was carried out to determine if this liver cell line contains cytochromes P450 that are immunochemically related to rainbow trout cytochromes P450 and to evaluate the inducibility upon exposure to TCDD and BNF. Characterization of the P450 system of this cell line is important in understanding the ability of these cells to metabolize foreign chemicals such as PAHs prior to their use in toxicological studies. The morphology, growth properties, and biochemical characteristics of this cell line were described in great detail in a separate paper (P. Collodi *et al.*, submitted for publication).

MATERIALS AND METHODS

Chemicals. Culture media (Leibovitz's L-15, Dulbecco's modified Eagle's, and Ham's F12 media) were purchased from GIBCO BRL (Gaithersburg, MD). 7-Ethoxyresorufin was obtained from Molecular Probes, Inc. (Eugene, OR). Resorufin, BNF, and unlabeled dimethylbenz[a]anthracene (DMBA) were purchased from Sigma (St. Louis, MO). 7,12-Dimethyl[G-³H]benz[a]anthracene ([³H]DMBA) was purchased from NCI Radiochemical Repository, Chemsyn Science Laboratories (Lenexa, KS). TCDD was purchased from ANALABS (New Haven, CT).

Cell culture. The conditions for establishing the zebrafish liver cell line (as outlined below) were the same as those described by P. Collodi *et al.* (manuscript submitted for publication). Adult zebrafish (obtained from the aquatic facility of the Marine/Freshwater Biomedical Sciences Center, Oregon State University), averaging 4.5 cm in length and 0.5 gm in weight, were killed, sterilized with 70% ethanol, and rinsed in sterile phosphate-buffered saline (PBS). Livers, 5–10 mg, dissected from two or three fish were combined and incubated 5 min at room temperature in 3 ml of trypsin/EDTA (0.02% trypsin, 1 mM EDTA in PBS, pH 7.2). The tissue was then gently dissociated by pipeting to form a suspension of individual cells and small cell aggregates. The cells were collected by centrifugation (500g), washed once with LDF culture medium (50% Leibovitz's L-15, 35% Dulbecco's modified Eagle's, and 15% Ham's F12 media supplemented with sodium bicarbonate (0.15 mg/ml), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.2), penicillin (200 international units/ml), streptomycin sulfate (200 µg/ml), and ampicillin (25 µg/ml) (10), and finally resuspended in 1 ml of LDF. The cell suspension was then added to a 35-mm tissue culture dish and the dish was allowed to sit undisturbed at room temperature for 30 min to enable individual cells and cell aggregates to attach to the plastic. The viability of the cell suspensions at this point could not be determined with accuracy because many of the cells were in aggregates, a condition important for the attachment of the cells to the plastic. An additional milliliter of LDF was gently added to the dish along with the following supplements: 5% fetal bovine serum, 10 µg/ml bovine insulin, 0.5% trout serum, 20 ng/ml mouse epidermal growth factor (EGF), and 40 µg/ml trout embryo extract. Trout serum and embryo extract were prepared as previously described (10, 12). Individual dishes were wrapped in parafilm to prevent drying and incubated at 26°C. Culture medium was replaced once a week.

After 2 to 3 weeks the cells grew to form a confluent monolayer consisting primarily of fibroblastic and epithelial cell types. Regions of the monolayer culture consisting exclusively of epithelial cells were removed from the dish using a plastic cloning ring. Fifty microliters of trypsin/EDTA was added to the ring and after 1 to 2 min approximately 100 cells inside the ring were dislodged by pipeting and transferred to a single well of a 96-well tissue culture plate. The trypsin was diluted with 150 µl LDF and the cells were allowed to attach for approximately 20 min after which 150 µl of the medium was gently removed and replaced with fresh LDF containing FBS, EGF, trout serum, and embryo extract. After 12 h, the medium was again changed to remove any residual trypsin. Medium was then changed once a week. Cultures which grew to form a

homogeneous monolayer of epithelial cells were expanded to a 24-well plate and eventually to a 75-cm² flask. The cells used for the experiments described had undergone a minimum of 20 population doublings in culture. A photograph of a representative zebrafish liver cell culture used in the studies described in this paper is shown in Fig. 1.

Treatment of the cultures and subcellular fractionation. Confluent zebrafish liver (ZF-L) cells grown in 175-cm² culture flasks (approximately 5×10^6 cells/flask) were exposed to culture medium containing TCDD (0.1, 1.0, and 10 nM), BNF (0.036, 0.36, and 3.6 µM), or DMSO (0.01% as vehicle control). After incubation at 26°C for 24 or 48 h, the cells were washed twice with phosphate-buffered saline (PBS) and scraped after the addition of Buffer A (50 mM Tris acetate buffer, pH 7.5, containing 20% glycerol and 0.1 mM EDTA) to the culture flasks. The cells were collected by centrifugation and the pellets were resuspended in Buffer A for homogenization using a Dounce homogenizer. The cell homogenates were stored at -80°C before use.

For subcellular fractionation, the cell pellets were homogenized in 50 mM Tris acetate buffer, pH 7.5, containing 0.1 M potassium chloride, 1 mM EDTA, and 0.1 mM PMSF (phenylmethylsulfonyl fluoride). The homogenates were first centrifuged at 600g for 10 min and the resulting supernatant was centrifuged at 8700g for 15 min. The 8700g supernatant was centrifuged at 105,000g for 90 min. The 600g pellet (nuclei), 8700g pellet (mitochondria), and 105,000g pellet (microsomes) were rehomogenized in Buffer A prior to storage at -80°C.

Measurement of catalytic activities. EROD activity in subcellular fractions from ZF-L cells was determined by the method of Prough *et al.* (13). DMBA hydroxylase activity was determined using [³H]DMBA as substrate by the method of DePierre *et al.* (14). All incubations were performed at 30°C. The metabolites of [³H]DMBA were analyzed by HPLC (15) using a Zorbax ODS column (4.6 × 250 mm) connected to a Radiomatic detector. Protein content was determined by Lowry *et al.* (16) using bovine serum albumin (BSA) as a standard and appropriate dilutions of Buffer A as reagent blanks to correct for the absorbance given by Tris.

Inhibition of EROD and DMBA hydroxylase activities by the P4501A1 inhibitor α -naphthoflavone (ANF) and by antibodies to trout P4501A1 was performed on homogenates of liver cells previously treated with 1.0 nM TCDD for 48 h. Homogenates were preincubated with ANF (dissolved in DMSO) for 5 min or with antibodies for 10 min at room temperature before the addition of other components of the reaction mixture. The catalytic activities in the presence of DMSO (for inhibition of ANF) or antibodies from nonimmunized rabbits (for inhibition by anti-trout P4501A1 IgG) were expressed as 100% for use in estimating the inhibitory effect of ANF and the anti-P4501A1 antibody.

Immunoblotting. Immunoblot (Western) analysis was used to detect the presence of P450 forms in ZF-L cells that are homologous to trout P450s. Subcellular fractions, separated by electrophoresis in 8% SDS-polyacrylamide gels, were transferred to nitrocellulose (17). After blocking with 2% BSA, the nitrocellulose sheets were exposed to primary antibody (antibodies to trout P450 LMC1, LMC2, LMC3, LMC5, or P450 1A1) (18, 19) and then to ¹²⁵I-protein A for detection of immunoreactive bands on a Kodak XAR film.

RESULTS

Morphological and Biochemical Characteristics

As stated earlier, the morphology, growth properties, and functional and biochemical characteristics of the ZF-L cells have been described in a separate communication (P. Collodi *et al.*, submitted for publication). The cells were exclusively of liver epithelial origin (Fig. 1) and exhibited a hypodiploid karyotype with a modal chromosome number of 47 ($2n = 50$). The cells also exhibited enzyme activities of differentiated liver tissue including aspartate

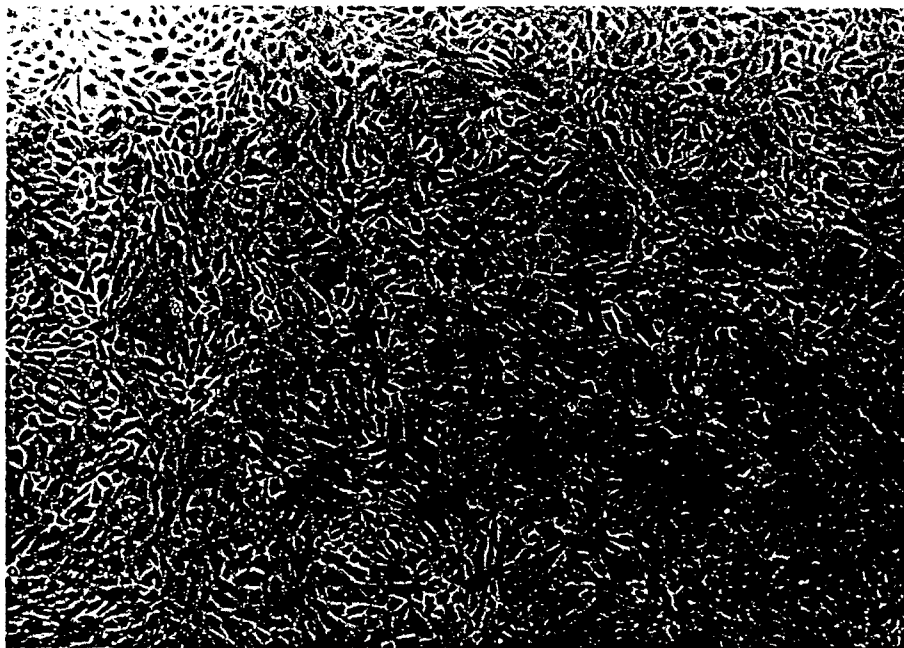


FIG. 1. Photomicrograph of zebrafish liver cells (100X) grown in LDF medium supplemented with insulin (10 μ g/ml), fetal bovine serum (5%), trout serum (0.5%), trout embryo extract (40 μ g/ml), and mouse epidermal growth factor (10 ng/ml).

transaminase, alkaline phosphatase, and glucose-6-phosphatase.

Catalytic Activities

DMSO-treated ZF-L cells displayed no EROD activity (Table I). Addition of TCDD to the culture medium resulted in a modest increase in EROD activity (75 to 117 pmol/min/mg protein) at all the concentrations used (0.1, 1.0, and 10 nM TCDD). No dose-related increase in EROD activity was observed, indicating that the lowest concentration of TCDD used (0.1 nM) was already saturating in terms of inducing EROD activity. In the cells exposed to 10 nM TCDD, EROD activity remained elevated after refeeding the cells with fresh medium (no TCDD) for 7 days. In contrast, addition of BNF to the culture medium did not increase EROD activity of the cells at 48 h when used at concentrations (0.036, 0.36, or 3.6 μ M) known to induce EROD activity in primary cultures of trout hepatocytes (8). Exposure of the cells to 0.36 μ M BNF for 24 h also did not increase EROD activity (Table I).

In addition to EROD activity, DMBA hydroxylase activity was used as a marker for induction of trout P4501A1-related enzymes in ZF-L cells. DMBA hydroxylase activity has been shown earlier to be induced by BNF in trout liver (20). As shown in Table I, DMBA hydroxylase activity of ZF-L cells was induced by TCDD but not by BNF and was barely detectable in DMSO-treated (controls) cells. Induction of DMBA hydroxylase activity was greatest at TCDD concentration of 1.0 nM and the induced activity persisted for at least 7 days after

withdrawal of the TCDD (10 nM) from the culture medium.

The intracellular localization of TCDD-induced EROD activity in ZF-L cells is shown in Table II. EROD activity was present in all fractions, the lowest and highest activities being in the nuclear (600g pellet) and microsomal fractions (105,000g pellet), respectively. No EROD activity was detected in any fraction derived from the DMSO-treated cells.

The subcellular distribution of TCDD-induced DMBA hydroxylase activity followed that of EROD in that the microsomes had the highest activity. All subcellular fractions from DMSO-treated cells displayed low but measurable DMBA hydroxylase activity (Table II). DMSO itself had no effect on EROD or DMBA hydroxylase activity of ZF-L cells (data not shown).

Results obtained in experiments with ANF and anti-trout P4501A1 IgG as inhibitors of catalytic activities of homogenates of ZF-L cells treated with 1.0 nM TCDD for 48 h are presented in Table III. At concentrations of 1 and 10 μ M, ANF completely inhibited EROD activity of the TCDD-treated cells. In contrast, DMBA hydroxylase activity was partially inhibited at 1 μ M concentration of ANF (54% inhibition) but complete inhibition was observed at 10 μ M concentration of ANF.

Anti-trout P4501A1 IgG inhibited EROD activity by 83% using antibody concentration of either 1 or 2 mg/mg of protein of TCDD-treated cells. However, the same concentrations of antibody only had a slight inhibitory effect (14% inhibition) on DMBA hydroxylase activity of the TCDD-treated cells.

TABLE I

Effects of BNF and TCDD on Ethoxyresorufin *O*-Deethylase (EROD) and 7,12-Dimethylbenzanthracene Hydroxylase (DMBA-OH) Activities of Homogenates of Cultured Liver Cells from Zebrafish^a

Treatment	Concentration	EROD	DMBA-OH
		pmol/min/mg protein	
Experiment A ^b			
DMSO	0.01%	0	1.2
BNF	0.036 μ M	0	0.70
	0.36 μ M	0	0.66
	3.6 μ M	0	0.72
Experiment B ^b			
DMSO	0.01%	0	1.07
TCDD	0.1 nM	110	5.66
	1.0 nM	117	13.55
	10.0 nM	75	9.04
Experiment C ^c			
DMSO	0.01%	0	1.05
TCDD	10.0 nM	114	11.13
Experiment D ^d			
DMSO	0.01%	0	0.97
BNF	0.36 μ M	0	0.85

^a Values are means of duplicate determinations for pools of four to six flasks per treatment.

^b Cells were exposed to BNF, TCDD, or DMSO (control) for 48 h and then harvested for analysis.

^c Cells were exposed to TCDD or DMSO for 48 h. Thereafter, the cells were washed with fresh medium and refed new medium without TCDD or DMSO for 7 days before termination.

^d Cells were exposed to BNF or DMSO for 24 h and then harvested for analysis.

HPLC analysis of the DMBA metabolites showed that the TCDD-exposed liver cells as homogenates or microsomes were highly active in converting DMBA mainly to DMBA-8,9-diol, 2-OH-DMBA, and to an unknown metabolite (Fig. 2). One of the other minor metabolites formed was the putative proximate carcinogen DMBA-3,4-diol. Only traces of diols and phenols were formed by the control (DMSO-treated) liver cells.

Immunoblotting

Figure 3 shows that anti-rainbow trout P4501A1 IgG cross-reacted strongly with a 54-kDa protein and to a lesser degree, with a 50-kDa protein in homogenates of ZF-L cells treated with TCDD for 48 h (lanes 2 to 4). Purified trout P4501A1 has an estimated minimum molecular weight of 58 kDa (lanes 5 and 6). The intensity of these two protein bands was greatest at the concen-

TABLE II

Subcellular Distribution of Ethoxyresorufin *O*-Deethylase (EROD) and DMBA Hydroxylase (DMBA-OH) Activities in Cultured Zebrafish Liver Cells Exposed to TCDD *in Vitro*^a

Subcellular fraction	EROD		DMBA-OH	
	DMSO	TCDD	DMSO	TCDD
Cell homogenate	0	99	1.07	9.04
Nuclei (600g pellet)	0	68	0.60	3.20
Mitochondria (8700g pellet)	0	72	1.20	9.00
Microsomes (105,000g pellet)	0	148	1.43	35.2

^a Cells were exposed to TCDD (10 nM) or DMSO (0.01%) for 48 h and then harvested for analysis. Values are means of duplicate determinations for pools of six flasks per treatment. Enzyme activities are expressed as pmol/min/mg protein.

tration of 1.0 nM TCDD. These two bands were not detected in DMSO-treated (Fig. 3, lane 1) or BNF-treated (Fig. 3, lanes 7–9) ZF-L cells after 48 h of exposure. Exposure of the cells to BNF (0.36 μ M) also did not induce the 54- or 50-kDa protein (data now shown). Induction of the proteins by TCDD persisted for 7 days following the withdrawal of the agent from the culture medium (data not presented).

The 54-kDa protein was primarily localized in microsomes from ZF-L cells (Fig. 4, lane 8) although the mitochondrial (Fig. 4, lane 4) and nuclear (Fig. 4, lane 10) fractions also showed the 54-kDa protein with lower intensity. The TCDD-induced 50-kDa protein from ZF-L cells was present as a faint band in mitochondrial, mi-

TABLE III

Effects of α -Naphthoflavone (ANF) and Anti-Trout P4501A1 Antibodies (Anti-1A1) on Ethoxyresorufin *O*-Deethylase (EROD) and 7,12-Dimethylbenzanthracene Hydroxylase (DMBA-OH) Activities of Homogenates of TCDD-Treated Zebrafish Liver Cells in Culture^a

Inhibitor	Activity (% of control)	
	EROD	DMBA-OH
ANF		
1 μ M	0	46
10 μ M	0	0
Anti-1A1	17	86
1 mg IgG/mg protein	17	86
2 mg IgG/mg protein	17	86

^a The reaction mixture was preincubated with ANF (in DMSO) for 5 min or with anti-1A1 IgG (1 or 2 mg IgG per milligram of homogenate protein) for 10 min at room temperature before NADPH was added to initiate the reaction. Values are percentages of activities observed in the presence of an equal volume (5 μ l) of inhibitor vehicle (DMSO) or of an equal quantity of nonimmune IgG.

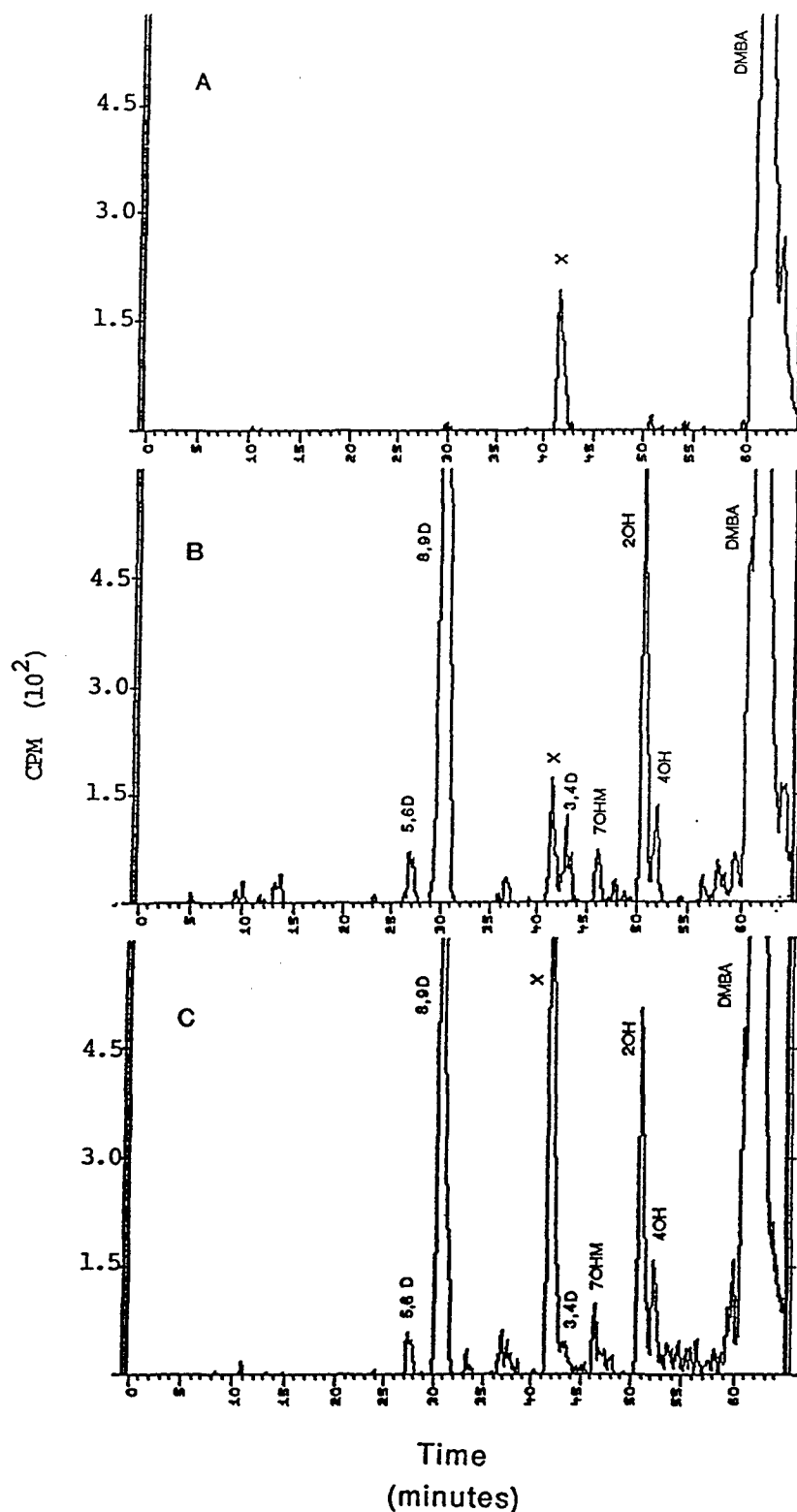


FIG. 2. HPLC separation of the metabolites of [^3H]DMBA by DMSO-treated cell homogenates (A), by TCDD-treated cell homogenates (B), and by microsomes from TCDD-treated cells (C). Tentative identification of metabolites was made by coelution with known standards as follows: 5,6D, *trans*-5,6-dihydroxy-5,6-dihydro-7,12-dimethylbenz[*a*]anthracene; 3,4D, *trans*-3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenzanthracene; 7OHM, 7-hydroxymethyl-12-methylbenz[*a*]anthracene; 2OH, 2-hydroxy-7,12-dimethylbenz[*a*]anthracene; and 4OH, 4-hydroxy-7,12-dimethylbenz[*a*]anthracene. Metabolite X is not identified.



FIG. 3. Immunoblot of homogenates (40 µg/lane) from DMSO (control)-, BNF-, and TCDD-treated zebrafish liver cells in culture probed with rabbit antitrat P4501A1 IgG. Lane 1, DMSO (0.01%)-treated cells; lane 2, TCDD (0.1 nM)-treated cells; lane 3, TCDD (1.0 nM)-treated cells; lane 4, TCDD (10 nM)-treated cells; lane 5, purified trout P4501A1, 0.25 pmol; lane 6, purified trout P4501A1, 0.5 pmol; lane 7, BNF (0.036 µM)-treated cells; lane 8, BNF (0.36 µM)-treated cells; and lane 9, BNF (3.6 µM)-treated cells.

crossosomal, and nuclear fractions. When the blots were probed with anti-trout P450 LMC2 antibody, no cross-reacting proteins were found in the ZF-L cells treated with DMSO, BNF, or TCDD (Fig. 5). The trout P450 LMC2 antibody recognized a single band (54 kDa) in trout liver microsomes (Fig. 5, lane 10) with the same mobility as the purified trout P450 LMC2 standard (Fig. 5, lane 6). Antibodies to other trout P450s (P450 LMC1, LMC3, LMC4, and LMC5) did not cross-react with any protein in DMSO-, BNF- or TCDD-treated ZF-L cells (data not shown).

DISCUSSION

This study demonstrates that untreated ZF-L cells have no detectable P4501A1 protein but, upon exposure to TCDD, displayed two protein bands (54 kDa and 50 kDa) that are recognized by anti-trout P4501A1. No proteins were recognized by antibodies to trout P450 LMC2 with an M_r of 54 kDa or by antibodies to other trout P450s including P450 LMC1 with an M_r of 50 kDa (18), suggesting that P450 homologous to the constitutive forms of trout P450 are not present in ZF-L cells. However, P450 LMC2 has been demonstrated in the liver of intact zebrafish by Western blot analysis and by immunohistochemical techniques (21). Thus, P450 LMC2 may have been lost in ZF-L cells during culture or the P450 was reduced to a level that was below the detection limits in the Western blot assay.

The TCDD-induced 54-kDa protein recognized by anti-trout P4501A1 IgG was also observed in primary cultures of TCDD-treated zebrafish hepatocytes (11), in the liver of intact zebrafish exposed to TCDD (P. Collodi *et al.*, manuscript in preparation) and in TCDD-treated zebrafish haploid and diploid-derived cells in culture (10).

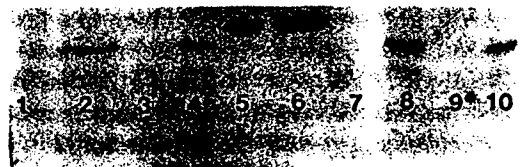


FIG. 4. Immunoblot of subcellular fractions (20 µg/lane) of zebrafish liver cells treated with DMSO (0.01%) or TCDD (10 nM) for 2 days before termination. The blot was probed with anti-trout P450 1A1 IgG. Lane 1, DMSO-treated cell homogenate; lane 2, TCDD-treated homogenate; lane 3, DMSO-treated mitochondria; lane 4, TCDD-treated mitochondria; lane 5, purified trout P4501A1, 0.125 pmol; lane 6, purified trout P450 1A1, 0.25 pmol; lane 7, DMSO-treated microsomes; lane 8, TCDD-treated microsomes; lane 9, DMSO-treated nuclei; and lane 10, TCDD-treated nuclei.

These results suggest that TCDD induces an identical protein (54 kDa) both *in vitro* and *in vivo* in zebrafish tissues. The 50-kDa was not detected in the haploid or diploid cells, but was present in the liver of the TCDD-induced zebrafish *in vivo* (P. Collodi *et al.*, in preparation). However, other workers (21) reported only one band (54 kDa) in the liver of TCDD-treated zebrafish. It is possible that there could be strain differences or genetic polymorphism in the expression of P4501A in zebrafish as with other species like tomcod (22).

Liver microsomes from several fish species treated with BNF have been immunoblotted using polyclonal antibodies against P4501A1 from rainbow trout and cod and monoclonal antibodies against scup P4501A1 (23). The apparent molecular weight of the cross-reacting proteins ranged from 54 kDa (scup) to 59 kDa (trout). There was only one major band observed in each sample recognized by the antibody probes. In ZF-L cells, however, two immunoreactive bands, 54 and 50 kDa, were recognized by anti-trout P450 1A1 IgG, indicating that two possible forms of P450 may be induced by TCDD in these cells. The more intense 54-kDa band and the less intense 50-kDa band were observed in cell homogenates, microsomes,

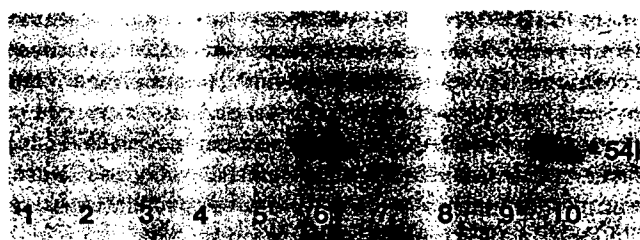


FIG. 5. Immunoblot of homogenates (40 µg/lane) from DMSO (control)- and BNF- and TCDD-treated zebrafish liver cells in culture probed with anti-trout P450 LMC2 IgG. Lanes 1 and 2, DMSO-treated cells; lane 3, TCDD (0.1 nM)-treated cells; lane 4, TCDD (1.0 nM)-treated cells; lane 5, TCDD (10 nM)-treated cells; lane 6, purified trout P450 LMC2, 0.5 nmol; lane 7, BNF (0.036 µM)-treated cells; lane 8, BNF (0.36 µM)-treated cells; and lane 9, BNF (3.6 µM)-treated cells. Lane 10 has 2 µg of untreated trout liver microsomes for comparison.

mitochondria, and nuclei (Fig. 4). There is a possibility that the 50-kDa immunoreactive protein could be a product of proteolytic cleavage of the 54-kDa protein assuming that PMSF was ineffective in inhibiting this proteolysis during homogenization and subcellular fractionation. A much more intensive investigation requiring protein purification, N-terminal amino acid sequence determination, or cDNA cloning is needed to establish beyond doubt that two separate forms of P450 are induced by TCDD in ZF-L cells.

The appearance of the two P450A-like proteins in all subcellular fractions of TCDD-treated zebrafish liver cells indicates two possibilities. First, the conventional method of subcellular fractionation probably is not suitable for cultured zebrafish liver cells such that the mitochondrial and nuclear fractions are contaminated by the microsomal fraction. Alternatively, the TCDD-induced protein(s) is indeed located in all fractions. Further work involving immunohistochemistry is needed to confirm the latter possibility. However, the induction of proteins with trout P450A1-like activity in ZF-L cells is clearly demonstrated by the increase in microsomal EROD and DMBA hydroxylase activities after treatment of the cells with TCDD.

Aside from the immunoblot studies, evidence suggesting the presence of two TCDD-inducible P450 enzymes in ZF-L cells was provided by the studies with the selective inhibitors of trout P450A1 (anti-P450A1 IgG and ANF). At a concentration of 1 μ M, ANF completely inhibited EROD activity whereas DMBA hydroxylase activity was inhibited only by 54%. Anti-trout P450 IgG at concentrations of 1 to 2 mg IgG per milligram of protein of cell homogenates inhibited EROD activity by 83% but DMBA hydroxylase activity was inhibited only slightly (14% inhibition) at these concentrations of antibody. These results suggest that EROD and DMBA hydroxylase activities may be mediated by two separate forms of P450.

The use of substrates associated with rodent P450A2 to demonstrate the presence of a P450A2 isozyme in ZF-L cells may not be valid or justified. For example, acetanilide, a substrate for rat P450A2, is an excellent substrate for trout P450A1 (19). We found that TCDD treatment (10 nM) of ZF-L cells produced a sevenfold increase in acetanilide 4-hydroxylase activity (unpublished observations) but it is not known whether this activity is mediated by a P450A1 or a related enzyme in ZF-L cells. Other substrates of rodent P450A2 such as 2-acetylaminofluorene may not be diagnostic substrates for a P450A2 in ZF-L cells for the same reason.

The inability of the ZF-L cells to respond to BNF in terms of increased protein or increased enzyme activity is a novel but intriguing finding. The concentrations of BNF used in the present study were those found to increase EROD activity, P450A1 protein, and P450A1 mRNA in primary cultures of trout hepatocytes in 24 to 48 h (7, 8). Exposure of the ZF-L cells to BNF for 24 or

48 h did not induce P450 activity or protein. Assuming that P450A1 induction in ZF-L cells is receptor-mediated, it is possible that the Ah receptor in these cells, unlike in trout hepatocytes, has strict requirements for recognition of structures of chemicals serving as its ligands. To examine this possibility, other known inducers of mammalian and fish P450A1 should be tested for their ability to induce P450A1 in ZF-L cells and their binding to the Ah receptor in these cells. The ZF-L cells may prove to be a unique nonmammalian model for studying structure-activity relationships in the induction of P450A1 *in vitro* and in understanding the role of the Ah receptor in P450 induction.

The metabolic activation of DMBA in mammalian cells involves the epoxidation of the compound by cytochrome P450, the hydration of the epoxides with epoxide hydrolase to form *trans*-dihydrodiols, and further epoxidation to form diol-epoxides (24, 25). The bay-region DMBA 3,4-diol-1,2-epoxide represents one of the proximate carcinogenic forms of DMBA (25). The formation of the DMBA-3,4-diol by rat liver microsomes is enhanced by pretreatment of rats with phenobarbital but not by 3-methylcholanthrene (26). The current study reveals that, unlike in rats, an inducer of P450A1, TCDD, induces the metabolic conversion of DMBA to DMBA-3,4-diol. TCDD treatment also increased the capacity of the ZF-L cells to convert DMBA to DMBA-8,9-diol, 2-hydroxy-DMBA, 4-hydroxy-DMBA, 7-hydroxy-12MBA, and metabolite (Fig. 2). The formation of the carcinogenic metabolites DMBA-3,4-diol and 7-hydroxy-12MBA (25-27) provide evidence that ZF-L cells, when exposed to TCDD, are capable of activating DMBA *in vitro*. The major DMBA metabolite, DMBA-8,9-diol, has relatively low mutagenic activity (26) but can induce malignant transformation in M2 fibroblasts (28). The consequence of increased metabolism of DMBA by ZF-L cells treated with TCDD is difficult to predict as a result of the differing biological activity of the various DMBA metabolites formed at different rates. This question could be resolved by utilizing TCDD-exposed ZF-L cells as the metabolic activating system in the Ames Salmonella assay for DMBA mutagenicity studies.

ACKNOWLEDGMENTS

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CULTURE OF CELLS FROM ZEBRAFISH (*BRACHYDANIO RERIO*) EMBRYO AND ADULT TISSUES

PAUL COLLODI, YUTO KAMEI*, TED ERNST, CRISTOBAL MIRANDA†, DONALD R. BUHLER†, AND DAVID W. BARNES

Department of Biochemistry and Biophysics
Environmental Health Sciences Center
Oregon State University, Corvallis, Oregon

†Department of Agricultural Chemistry
Oregon State University, Corvallis, Oregon

*Agribusiness Department
Sapporo Breweries, Ltd., Tokyo, Japan

The zebrafish is a popular model for studies of vertebrate development and toxicology. However, in vitro approaches with this organism have not been fully exploited because cell culture systems have been unavailable. We developed methods for the culture of cells from blastula-stage diploid and haploid zebrafish embryos, as well as cells from the caudal and pelvic fin, gill, liver, and viscera of adult fish. The haploid embryo-derived cells differentiated in culture to a pigmented phenotype and expressed, upon exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin, a protein that was immunologically and functionally similar to rainbow trout cytochrome P450IA1. Zebrafish cultures were grown in a complex basal nutrient medium supplemented with insulin, trout embryo extract, and low concentrations of trout and fetal bovine serum; they could not be maintained in conventional culture medium containing a high concentration of mammalian serum. Using calcium phosphate-mediated transfection, a plasmid constructed for use in mammalian cells was introduced into zebrafish embryo cell cultures and expressed in a stable manner. These results indicated that the transfection procedures utilized in mammalian systems can also be applied to zebrafish cell cultures, providing a means for in vitro alteration of the genotype and phenotype of the cells.

1. Address all correspondence to: David W. Barnes, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331. Tel: (503) 737-3200.

2. Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; EROD, 7-ethoxyresorufin; HDPDS, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; LDF, limit dilution factor; DMSO, dimethyl sulfoxide; ES, embryonal stem; PAH, polycyclic aromatic hydrocarbons; ZG, zebrafish gill; ZBF, zebrafish pelvic fin; ZV, Zebrafish viscera; ZCF, zebrafish caudal fin; ZEM, diploid blastula-derived.

INTRODUCTION

Fish are popular experimental organisms for studies of toxicology; it is economical to maintain sizable populations for large-scale exposures and many species display acute sensitivities to various xenobiotics (Schultz and Schultz, 1982a, 1982b; Babich and Borenfreund, 1991). Often such studies are conducted on young rainbow trout and other salmonids that are only available on a seasonal basis and require a year or more to become sexually mature (Hendricks et al., 1984; Bailey et al., 1984; Collodi et al., 1984). Recent attention has focused on the use of small aquarium species such as the medaka and zebrafish which grow rapidly, reach sexual maturity in approximately three months, and are tolerant of temperature and salinity (Hatanaka et al., 1982; Egami et al., 1981; Aoki et al., 1977; Bresch 1991; Bresch et al., 1990; Babich and Borenfreund, 1991).

Rapidly developing zebrafish embryos can be obtained at little expense and at any time of the year for laboratory investigations. Methods exist for producing homozygous embryos and clonal lines of fish, which allows for the identification of individual fish that possess nonlethal recessive mutations and thus allow for the maintenance of the mutation within a genetically homogeneous population (Streisinger et al., 1981). Methods also have been developed for derivation of transgenic zebrafish (Stuart et al., 1988, 1990). Approaches involving the genetic manipulation of zebrafish could lead to the derivation of strains highly sensitive to non-genotoxic carcinogens or environmental tumor promoters that would be useful for chemical screening.

Although *in vivo* studies of the effects of xenobiotics on various stages of the zebrafish life cycle (Bresch, 1991; Bresch et al., 1990; Nagel, et al., 1991; Dave and Xiu, 1991) have been informative, virtually no work has been directed to *in vitro* cell culture approaches to the study of zebrafish toxicology. This is probably the case because multipassage culture of zebrafish cell lines under conventional conditions with mammalian sera as a primary source of growth-stimulating factors has not been successful (Bols and Lee, 1991). As a result, little is known about the biochemical parameters that influence zebrafish embryonal cell growth and differentiation.

We and others have observed that some embryo-derived mammalian cells are growth-inhibited by mammalian sera (Loo et al., 1987, 1989; Rawson et al., 1991; Shirahata et al., 1990). Previously, we reported mitogenic activity in trout embryo extract that promotes growth of established fish cell lines and primary salmonid embryo cell cultures in medium containing little or no serum (Collodi and Barnes, 1990). In this paper we describe application of this approach to the culture of zebrafish cells. We report conditions supporting the long-term growth of cells derived from blastula-stage zebrafish embryos as well as in the culture of cells from zebrafish fin, liver, viscera, and gill.

We have also taken advantage of a useful aspect of the zebrafish system to produce cell cultures from haploid embryos. Fertilization of zebrafish eggs with UV-inactivated sperm

allows the production of haploid zebrafish embryos that undergo the early stages of development (Streisinger et al., 1981), well past the stages from which we could derive cell cultures. Haploid embryonal cell cultures may facilitate studies involving the inactivation of targeted genes. Clones could be derived that are specifically altered in a predetermined gene through targeted inactivation schemes and selections, providing useful lines for cell culture experiments (Kaufman et al., 1983; Ishino et al., 1990).

Mammalian embryonal cell cultures, such as 10T1/2 cells, have been used as a model to study the expression of genes coding for xenobiotic-metabolizing enzymes such as cytochrome P450 (Pottenger and Jefcoate, 1990). In this report we provide evidence that exposure of zebrafish embryo cell cultures to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) resulted in the expression of a new microsomal protein that was recognized by anti-rainbow trout P450IA1 IgG. The TCDD-treated cells also displayed TCDD-inducible 7-ethoxyresorufin O-deethylase (EROD) activity; an activity mediated by cytochrome P450IA1 and P450IA2 enzymes (Kelley et al., 1987). Our results suggest that zebrafish embryo-derived cultures may provide a means for *in vitro* toxicological studies of xenobiotics activated by inducible polycyclic aromatic hydrocarbon-metabolizing enzymes.

MATERIALS AND METHODS

Cell Culture

Synchronously developing zebrafish blastula-stage embryos for the initiation of cultures (diploid blastula-derived ZEM cultures and haploid blastula-derived ZEMH cultures) were obtained by *in vitro* fertilization. Zebrafish are photoperiodic in their breeding; fish were maintained on a 14 hr light/10 hr dark cycle; eggs and sperm were collected within 90 min after fish were exposed to light. Fish were anesthetized with 3-amino benzoic acid ethylester and sperm from 2 to 3 males collected in a capillary tube and stored on ice in approximately 50 μ l of Hank's solution. Sperm remain viable for up to 90 min. Eggs were collected from a gravid female (approximately 100 eggs/fish) by gently pressing on the ventral side of the fish. Eggs were mixed with sperm in a small petri dish and fertilization was initiated by the addition of 1 ml water to dilute the Hank's solution and activate the sperm. After 2 min, additional water was added to the fertilized eggs and embryos were allowed to develop undisturbed at 26°C (Streisinger et al., 1981).

Blastula-stage embryos were harvested 2 to 3 hr after fertilization and rinsed three times in the same basal nutrient medium used for cell culture (limit dilution factor medium [LDF], described below) and then rinsed once in a 0.5% bleach solution. Following three additional rinses in nutrient medium, the embryos were dechorionated in trypsin solution (0.2%, wt/vol, trypsin/1mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline, for approximately 10 min and the cells were dissociated by pipeting the embryos gently until a suspension of small cell aggregates was obtained. The cell aggregates were then collected by centrifugation and plated in 96-well tissue culture dishes (Costar). Cells pooled from 3 to 5 embryos were added to each well.

For generation of haploid blastula-derived cell cultures, sperm was UV-irradiated (29 cm from a 43 cm long Sylvania germicidal tube) 2 min before fertilizing the eggs (Streisinger et al, 1981), followed by the cell culture procedures described above. Eighty percent of the resulting embryos develop normally through gastrulation; all die before hatching.

Cells were grown in LDF medium (50% Leibovitz's L-15, 35% Dulbecco's modified Eagle's and 15% Ham's F12 media) (Gibco) supplemented with sodium bicarbonate (0.15 mg/ml), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2), penicillin (200 international units/ml), streptomycin sulfate (200 ug/ml), ampicillin (25 ug/ml), bovine insulin (10 ug/ml; Sigma), trout embryo extract (25 ug/ml), trout serum (0.1%), and fetal bovine serum (FBS) (1%) (Gibco). After approximately 7 days the cells were trypsinized and passaged into a 24-well tissue culture plate and eventually expanded into 25 cm² flasks. Medium was changed once each week.

Primary cultures from adult tissues (zebrafish gill, pelvic fin, caudal fin, and viscera) were derived in the same medium by washing the dissected tissues 3 times in nutrient medium, mincing, and digesting the mince in trypsin while pipeting to dissociate the cells. Fish were approximately one year old. The dissociated cells and cell aggregates were plated into 25 cm² tissue culture flasks and the medium was changed once a week. When a confluent monolayer had formed (approximately 2 weeks) the cells were trypsinized and passaged.

Cell Transfections

The pSV2-neo plasmid (Southern and Berg, 1982) was obtained from the American Type Culture Collection. For transfections, 25 ug of plasmid DNA in 0.5 ml to 0.25 M calcium chloride was added dropwise with constant mixing to 0.5 ml HEPES-buffered saline (250 mM HEPES, pH 7.0) containing 1.8 mM sodium phosphate. The resulting precipitate-containing suspension was incubated 30 min at room temperature, and the suspension sheared twice through a 25 gauge needle. The suspension was then added directly to a flask containing approximately 10⁶ cells that had undergone a medium change approximately 4 hr previously. Medium was changed to remove the precipitate 6 hr after its addition. Cells were allowed to grow 7 days before addition of the antibiotic G418 (Geneticin) at a concentration of 500 ug/ml. Colonies obtained from the selection were isolated and expanded.

Preparation of Trout Embryo Extract

Approximately 100 Shasta strain trout embryos (21-day old, developed at 10°C and stored frozen at 80°C) were thawed and pooled in 5-10 ml of basal nutrient medium. The embryos were homogenized 3 times for 15 sec each time on ice with a Tissuemizer cell homogenizer (Tekmar). The resulting homogenate was centrifuged for 5 min (15,000 x g) and the supernatant collected, avoiding the layer of lipid which formed on the top of the supernatant. After diluting (1:5) with basal nutrient medium, the supernatant was filter sterilized through a 0.4 um prefilter followed by a 0.2 um filter. Protein concentrations were determined by the method of Bradford (1976).

Preparation of Fish Serum

Fish serum was prepared from the blood of Shasta strain rainbow trout. After removing the cells by centrifugation, the plasma was allowed to clot at 4°C for 16 hr. Serum was separated from the clotted protein by centrifugation and sterilized by filtration (0.2 μ m filter). The serum was incubated at 56°C for 20 min before use in cell culture.

Growth Assays

Growth assays were conducted at 26°C in duplicate in 6-well (35-mm diameter) tissue culture plates (Falcon). Medium was changed every 5 days during the assay and the cells were grown in ambient air. Each plate was wrapped in Parafilm to prevent evaporation. For each assay, cells were plated at 5×10^4 /well in the appropriate medium and at the designated time a suspension of trypsinized cells in PBS was counted using a Coulter particle counter.

Karyotype Analysis

Rapidly growing cultures were incubated in colcemid (0.2 μ g/ml) for 9 hr. Cells were then trypsinized, centrifuged, and the pellet gently resuspended in 0.4% KC1 and incubated at room temperature (20 min). Following incubation, 1 ml fixative was added to halt the action of the hypotonic KC1. The cells were then fixed and washed 3 times in methanol/acetic acid (3:1). Chromosome spreads were prepared and stained in 3% Giemsa solution prepared in Sorenson's buffer, pH 6.8, and karyotype analysis was performed on approximately 50 metaphases (Ernst et al., 1991). This procedure is derived from that used for mammalian cell cultures, and optimization for zebrafish cells (e.g., incubation time with hypotonic solution, KC1 concentration) has not yet been addressed.

Exposure to TCDD In Vitro and Evaluation of Enzyme Induction

Confluent haploid zebrafish embryo-derived (ZEMH) cells grown in 175-cm² culture flasks (approximately 10^7 cells/flask) were exposed to culture medium containing dimethyl sulfonide (DMSO) (control) or TCDD (10 nM) in DMSO. The final concentration of DMSO was 0.01%. After incubation at 26°C for 48 hr, the cells were scraped and collected by centrifugation. The pelleted cells were resuspended in 0.1 M Tris acetate buffer, pH 7.4 containing 0.1 M potassium chloride, 1 mM EDTA and 0.1 mM PMSF (alpha tolunesulfonylfluoride). The cells were disrupted by sonication and then homogenized using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g for 30 min and the resulting supernatant was recentrifuged at 105,000 g for 90 min. The microsomal pellet was resuspended in 10 mM Tris acetate buffer, pH 7.5, containing 20% glycerol and 0.1 mM EDTA.

Microsomes were subjected to SDS-polyacrylamide gel electrophoresis and fractionated proteins were transferred to nitrocellulose by electroblotting. Filters were exposed to primary antibody (antitrout P450IA1 IgG) and goat antirabbit IgG was conjugated with horseradish peroxidase with detection by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL). EROD activity of microsomes was measured according to the method of Prough et al., 1978. Microsomal protein was determined by the method of Lowry et al., 1951.

RESULTS

Derivation and Characteristics of Zebrafish CII Cultures

We have derived zebrafish cultures from diploid blastula (ZEM), haploid blastula (ZEMH) and from adult tissues: gill, pelvic fin, caudal fin, and viscera. The diploid blastula-derived cell line (ZEM) has been cultured continuously for more than 15 passages (approximately 40 population doublings) in LDF basal nutrient medium supplemented with sodium bicarbonate, insulin, trout embryo extract, trout serum and FBS. The doubling time of the embryo cells under these conditions was approximately 96 hrs. Growth of the embryo cells in LDF was superior to that achieved with several other basal nutrient media alone and in combination (Table 1).

TABLE 1. Growth of ZEM Cells in Basal Nutrient Medium Formulations and Mixtures

MEDIUM	CELLS/WELL
Dulbecco's modified Eagle's medium (DME)	7.6×10^4
Ham's F12 and DME (50:50 mixture)	2.1×10^4
Ham's F12 and DME (70:30 mixture)	4.8×10^4
Ham's F12 and DME (30:70 mixture)	2.0×10^5
Liebovitz' L-15 and DME (50:50 mixture)	5.2×10^5
Liebovitz's L-15, Ham's F12 and DME (10:7:3 mixture)	3.2×10^5
Liebovitz's L-15, DME and Ham's F12 (50:35:15 mixture)	7.7×10^5
Enriched RPMI-DME-F12 (50:25:25)	6.7×10^4

Cells were plated (5×10^4 per well) in 6-well (35-mm diameter) tissue culture plates as described in "Materials and Methods". A suspension of trypsinized cells in phosphate-buffered saline was counted 12 days after plating. Average variation of single determinations from the mean was less than ten percent. Each medium or mixture was supplemented with insulin (10 ug/ml), FBS (1%), trout serum (0.4%) and trout embryo extract (25 ug/ml). Enriched RPMI-DME-F12 mixture was obtained from Kyokuto Pharmaceutical, Tokyo.

Cell lines with similar growth rates were derived from caudal and pelvic fin, gill, and viscera (Figures 1 and 2). Cultures were also derived from adult zebrafish liver (Figure 2); these cells have been propagated in culture for approximately 5 population doublings. The growth rate was slow (doubling time greater than 4 days), but a high percentage of the cells comprising the culture were capable of proliferation *in vitro*. Fin-derived cells were fibroblastic in appearance, while liver-derived cultures were primarily epithelial. Viscera-derived cell cultures, initiated from liver-associated tissue that had been dissected free, appeared fibroblastic (not shown).

All of the cell lines required a low concentration (1-3%) of FBS for optimal growth; higher concentrations of FBS inhibited both ZEM and viscera cell growth, but not the growth of the fin-derived cell lines (Figure 3). For short-term culture of ZEM cells, beta-mercaptoethanol (10^{-4} M) could replace FBS, an advantage when a more defined medium is useful. All 5 cell lines exhibited a strong dependence for growth on a low concentration of trout serum (Figure 4). As little as 0.1% allowed optimal growth of ZEM Cells and, as with FBS, the cells were growth-inhibited by higher concentrations of trout serum. The viscera- and fin-derived cell lines were not inhibited by higher trout serum concentrations.

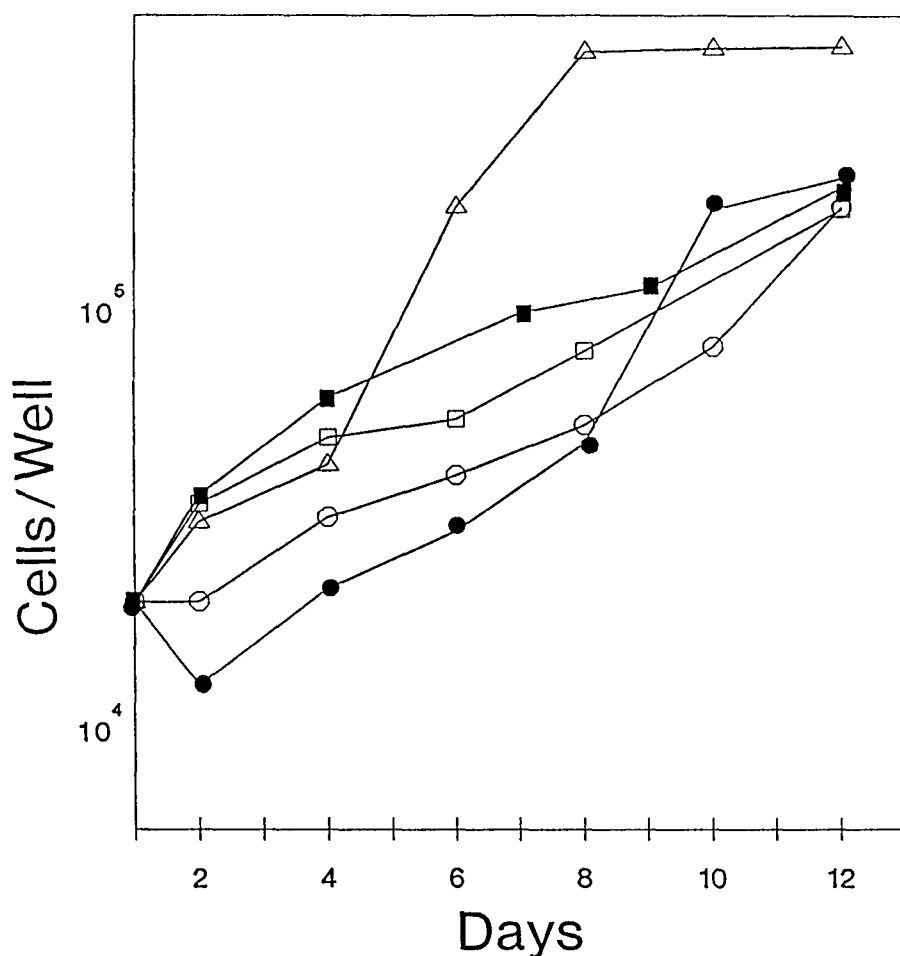


FIGURE 1. Zebrafish cell growth in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout serum 0.4%, and trout embryo extract (25 ug/ml). Cells were plated as described in Table 1 and counted on the days indicated. Average variation of single determinations from the mean was less than 10%. ZG (Δ); ZPF (●); ZCF (○); ZV (■); ZEM (□).

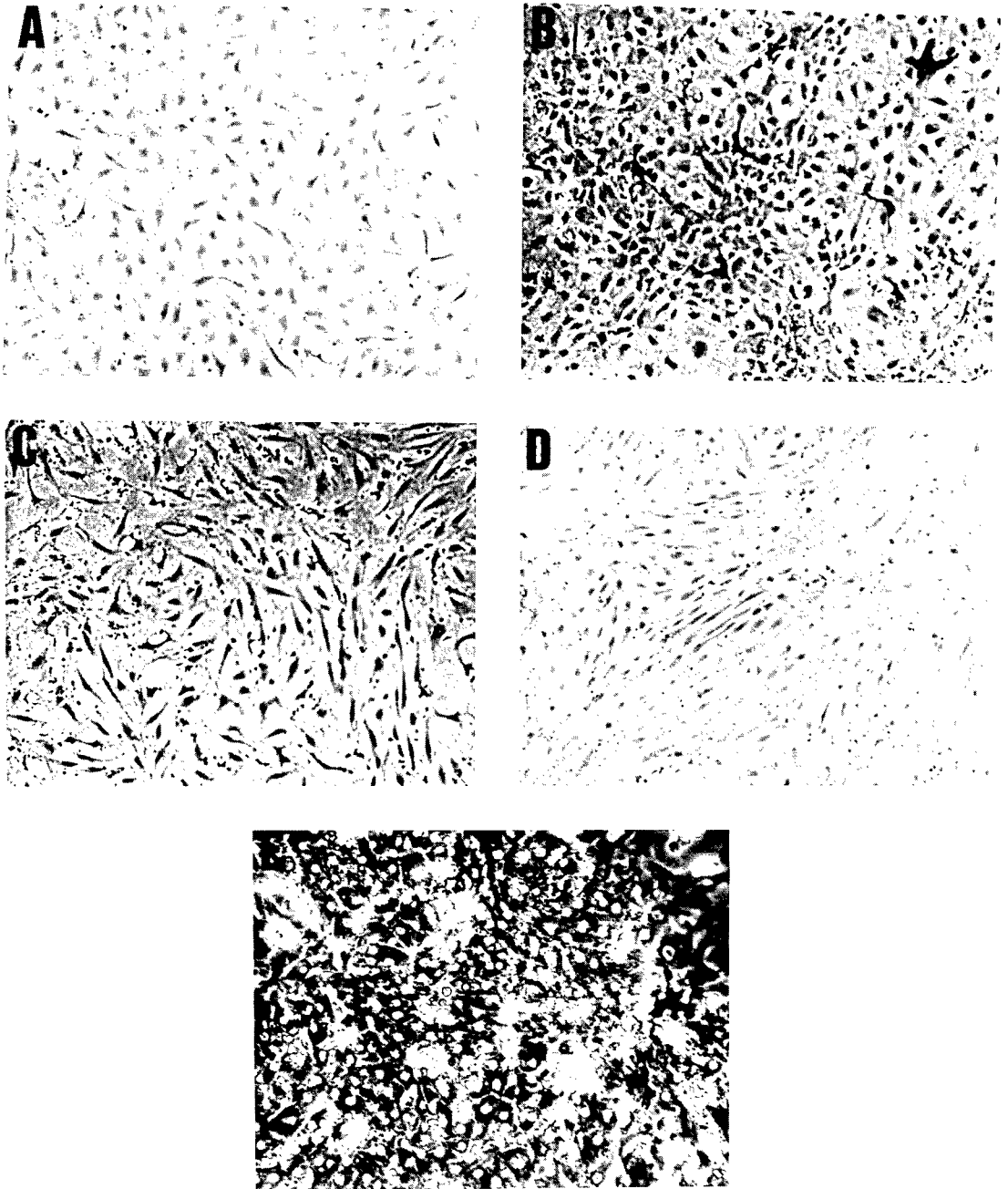


FIGURE 2. Photomicrographs of zebrafish cells grown in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout serum (0.4%), and trout embryo extract (25 ug/ml): A, ZEM; B, ZEMH; C, ZPF; D, ZCF; E, liver. All are 100X magnification except E (200X).

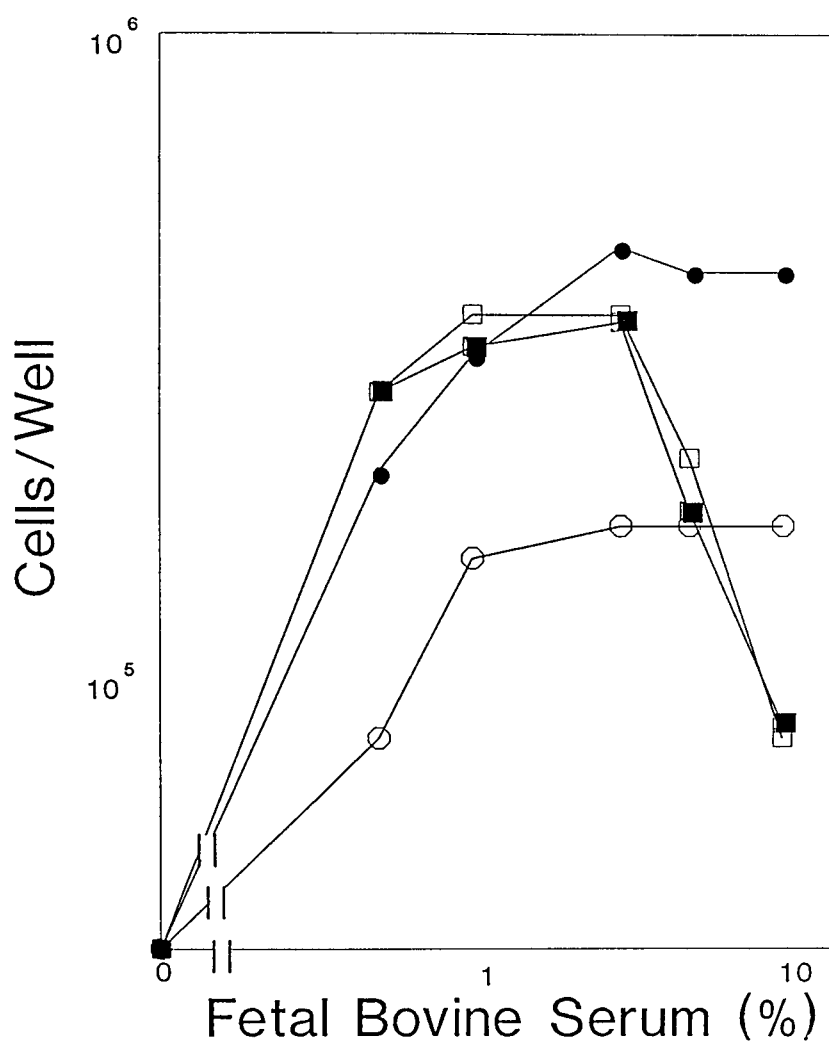


FIGURE 3. Effect of FBS on zebrafish cell growth. Cells were plated in LDF medium supplemented with insulin (10 ug/ml), trout serum (0.4%), trout embryo extract (25 ug/ml) and FBS (0 to 10%) as described in Table 1 and counted 12 days after plating. Average variation of single determinations from the mean was less than ten percent. ZEM (□); ZV (■); ZCF (○); ZPF (●).

Trout embryo extract and insulin were necessary to establish all of the cell lines in culture; without these factors cultures initiated from primary tissue stopped growing after a few days. After the cultures had been propagated for 15 or more population doublings *in vitro*, all but one of the cell types required only FBS and trout serum as supplements for optimal cell growth in a short-term experiment (Figure 5). Only caudal fin cells continued to acutely require supplementation with embryo extract for optimal growth under these conditions, although several of the cell types exhibited a continued requirement for the trout embryo-derived extracts for optimal growth in longer-term multipassage experiments.

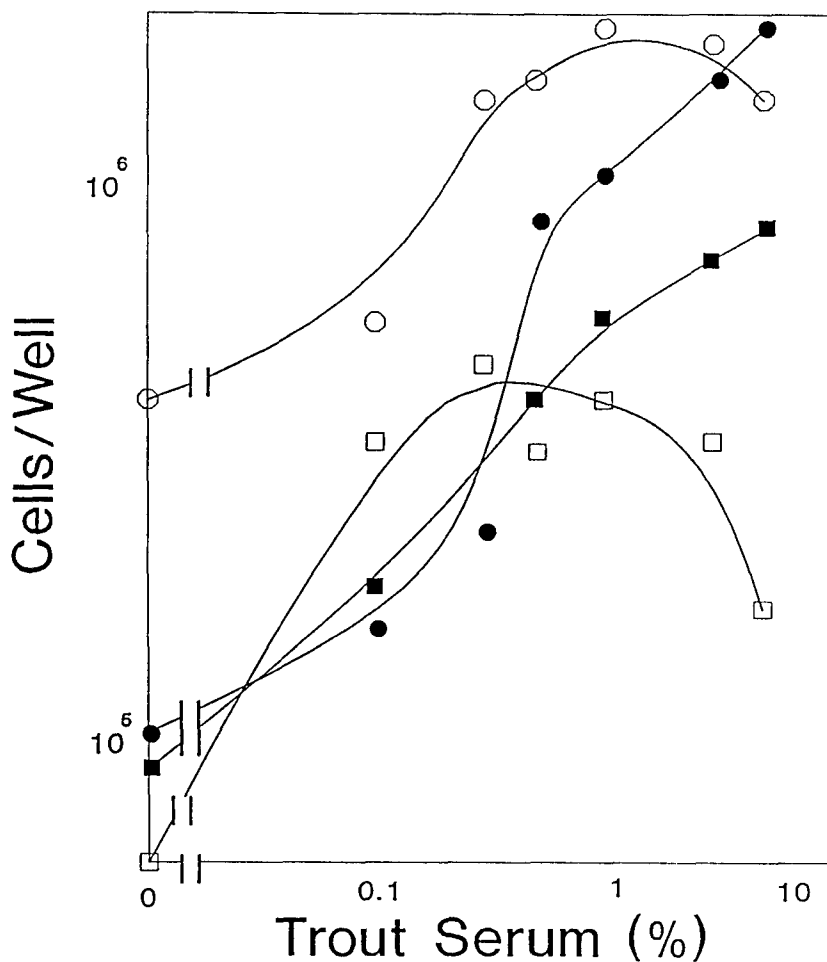


FIGURE 4. Effect of trout serum on zebrafish cell growth. Cells were plated in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout embryo extract (25 ug/ml), and trout serum (0 to 10% as described in Table 1 and counted 12 days after plating. Average variation of single determinations from the mean was less than ten percent. ZEM (□); ZV (■); ZCF (○); ZPF (●),

The basis for difference in trout embryo extract requirements for the two fin-derived cultures (caudal and pelvic) is not clear. Caudal fin cells were grown in culture for a similar number of generations as pelvic fin cells at the time of the experiment, indicating that the difference in response is not the result of a difference in general adaptation to *in vitro* conditions. The difference in behavior *in vitro* may reflect a difference in available tissue source at the time of preparation of the cells. Material for derivation of the fin cell cultures contained both ray and interray tissue, and these cultures were initiated from the mixture of cell types present in these areas.

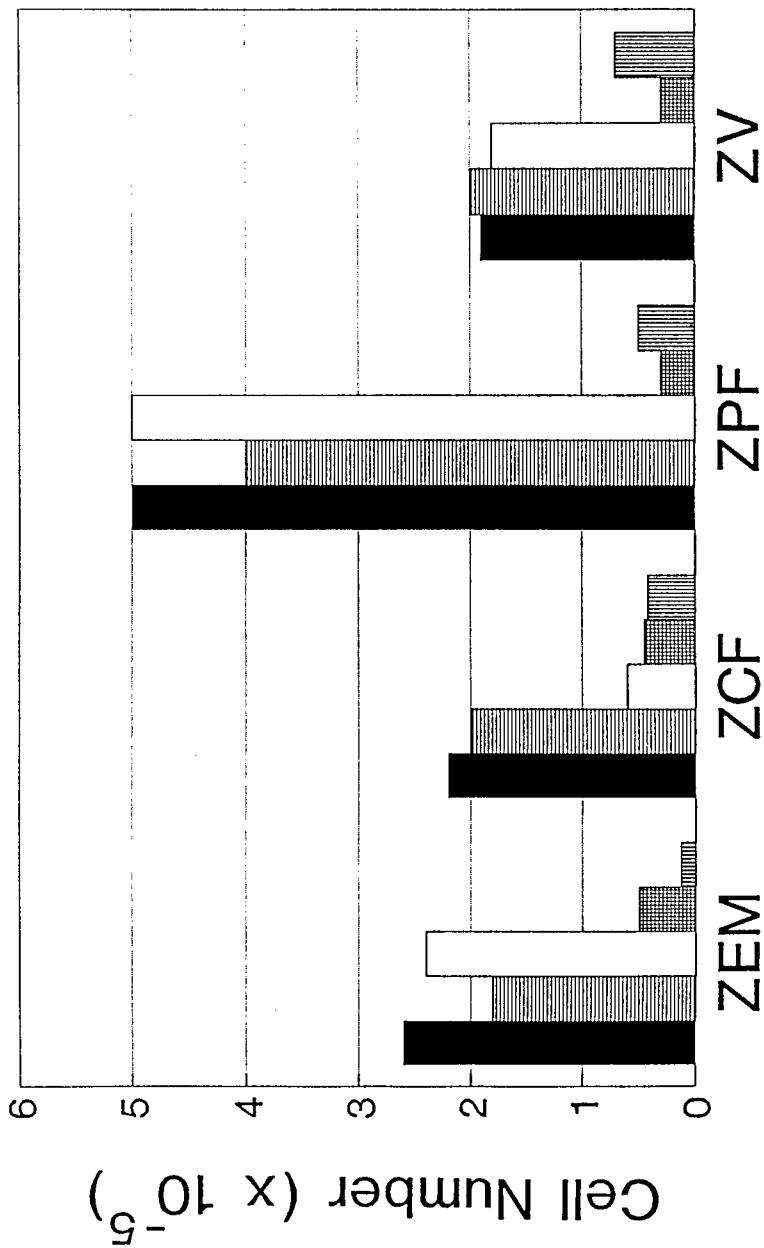


FIGURE 5. Effect of LDF medium supplements on zebra fish cell growth. Cells were plated in 6-well (35 mm-diameter) tissue culture plates as described in Table 1 in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout serum (0.4%) and trout embryo extract (25 ug/ml) (■) or medium from which insulin (▨), trout embryo extract (▩), trout serum (■), or FBS (▨) was individually omitted. Cells were counted 12 days after plating. Average variation of single determination from the mean was less than ten percent.

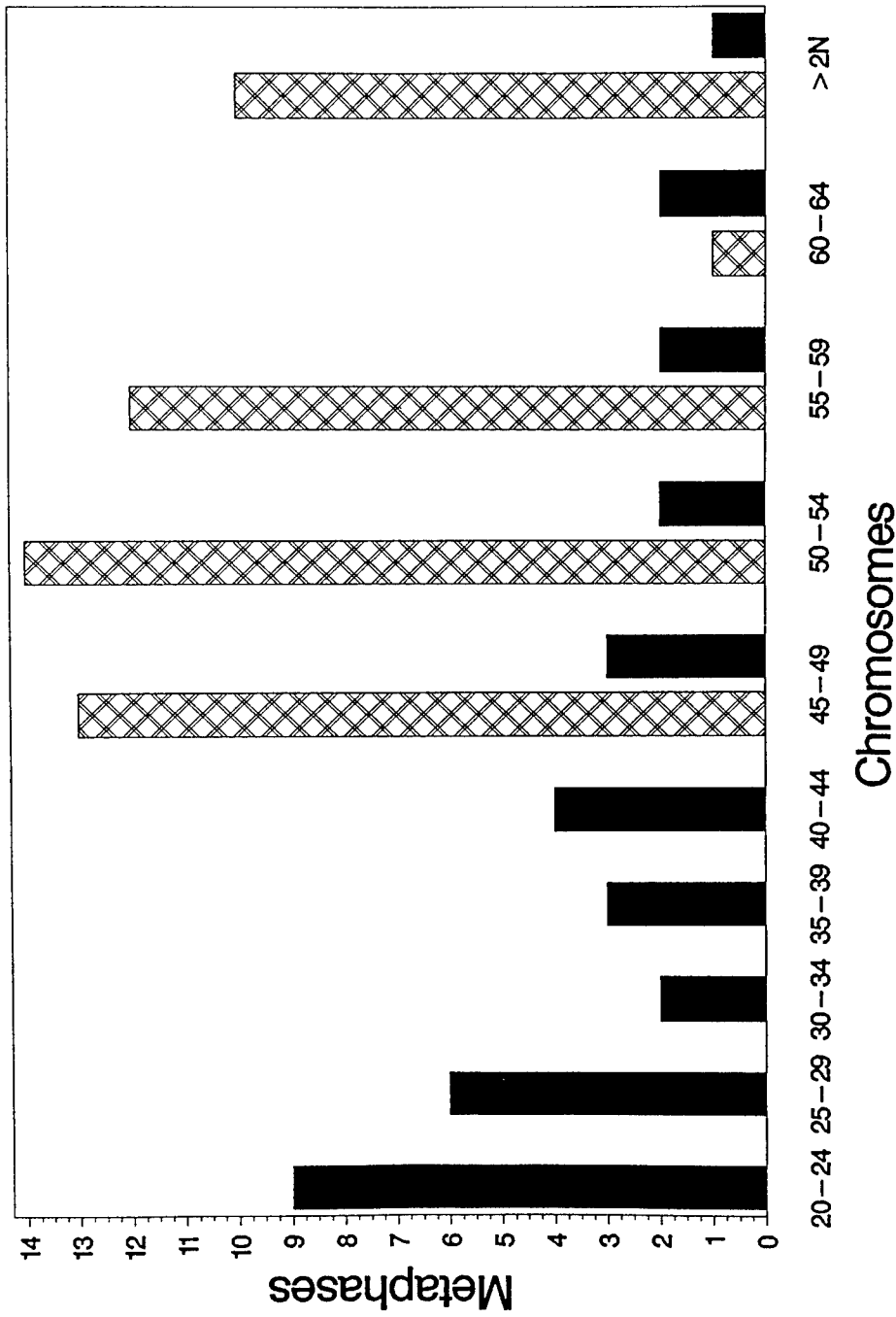


FIGURE 6. Karyotypic analysis of zebra fish cells. ZEM and ZEMH cells were grown and prepared for karyotyping as described in "Materials and Methods". The chromosome number distribution was obtained from 50 ZEM and 34 ZEMH metaphases. ZEM, crosshatched bars; ZEMH, solid bars.

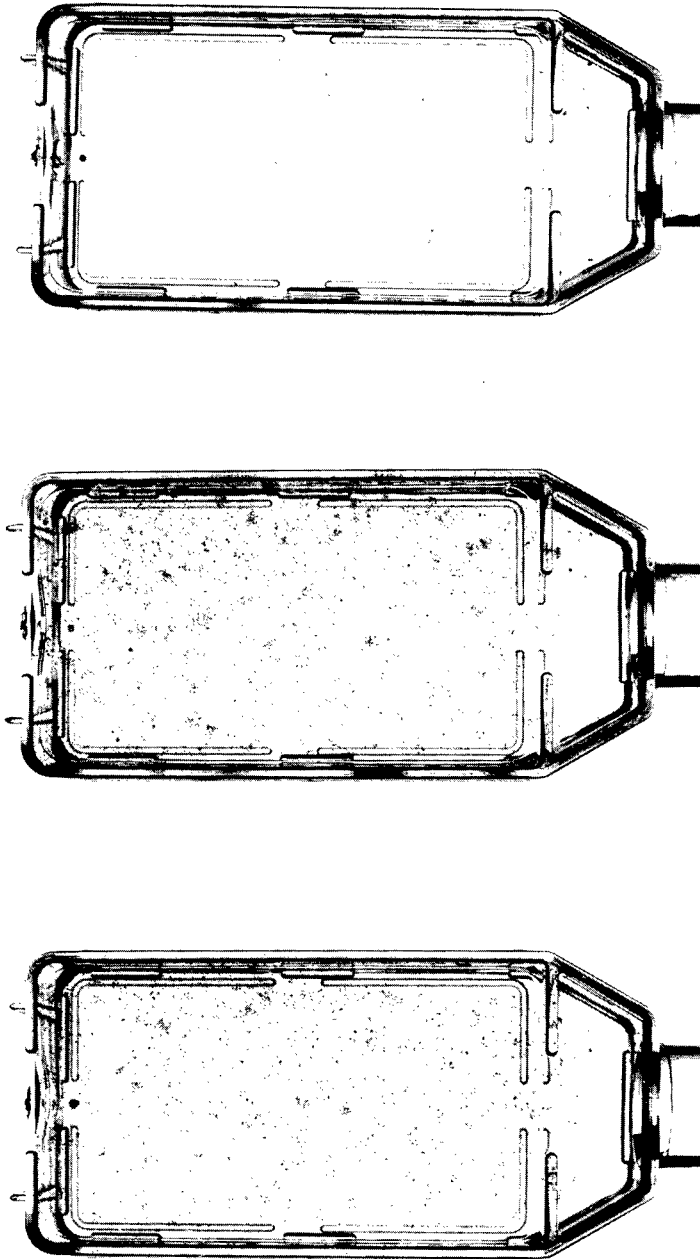


FIGURE 7. Growth of pSV2-neo transfected *ZEM* cells in G418. Following transfection and selection in G418 as described in "Materials and Methods", 10^4 cells were plated in 25 cm² tissue culture flasks and grown for 3 weeks in the presence or absence of 400 ug/ml G418. Colonies present in each flask were visualized by staining with crystal violet as described in "Materials and Methods". Nontransfected *ZEM* cells (top flask) and two pSV2-neo transfected clones are shown.

In addition to the ZEM cell line, a second embryo-derived cell line, ZEMH, was established from blastula-stage embryos developed from eggs fertilized with UV-inactivated sperm. Karyotype analysis of ZEMH cells revealed that after approximately 30 population doublings in culture, 79% of the population possessed a hypoploid chromosome number and 63% of these cells possessed the haploid chromosome number (Figure 6). The mode for the population was 24; the modal chromosome number for ZEM cells was 51. Diploid chromosome number for *Brachydanio rerio* is 50 (Endo and Ingalls, 1968). ZEMH cells were similar in appearance to ZEM cells, but were capable of differentiation in culture to a pigmented phenotype (Figure 2). These cells appear black in the light microscope and may be melanocytes, although other pigmented cells exist in the fish that also may appear black upon microscopic examination. Individual pigmented ZEMH cells appeared in dense cultures and represented less than 1% of the total cell population. Neither the haploid nor diploid embryo-derived cultures appeared transformed by morphological criteria associated with transformed mammalian cells in culture.

Transfection of ZEM cells

To demonstrate the potential of zebrafish cells in culture to express exogenous genes introduced into the cells by transfection, we isolated stable transfectants expressing the pSV2-neo plasmid containing the gene for bacterial aminoglycoside phosphotransferase under the influence of the simian virus 40 (SV40) early promoter and polyadenylation signal (Southern and Berg, 1982). This gene confers resistance to the antibiotic G418 and the plasmid construction is active in cell cultures derived from trout and salmon embryo and trout hepatoma (Helmrich et al., 1988). G418-resistant colonies appeared with a frequency of approximately one in 10^5 cells (Figure 7).

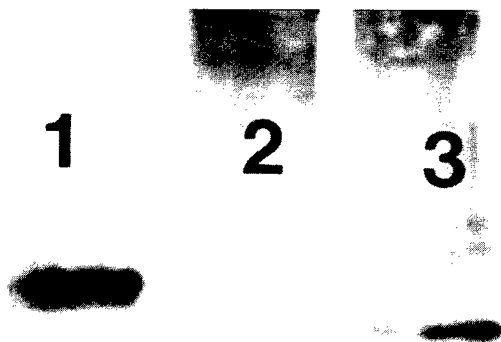


FIGURE 8. Immunoblot of liver microsomes from control and TCDD-treated ZEMH cells probed with anti-trout P450IA1 IgG. Procedures are described in Materials and Methods. Lane 1, purified trout P450IA1 (0.25 pmols); lane 2, control ZEMH (25 ug); lane 3, TCDD-treated ZEMH (25 ug).

Exposure of ZEMH Cells to TCDD

Immunoblotting (Western) blotting established that TCDD induced a microsomal protein in ZEMH cells that is recognized by anti-trout P450IA1 IgG. Figure 8 shows that microsomes derived from TCDD-treated ZEMH cells contained an immunoreactive band running slightly below that of purified trout P450IA1 ($M_r=60,000$), as with other fish species (Goksoyr et al., 1991). This band was not found in control (DMSO-treated) microsomes. The microsomes from TCDD-treated ZEMH cells also displayed low, but measurable EROD activity (12 pmol/min/mg protein), which was not exhibited by the control microsomes. Similar results were obtained when ZEM and liver-derived cultures were exposed to TCDD (Collodi et al., 1992b).

DISCUSSION

This paper describes the derivation and characterization of cell cultures initiated from blastula-stage zebrafish embryos (ZEM, ZEMH), and from several tissues of adult zebrafish. Our success in deriving the cultures was largely due to the use of a complex mix of basal nutrient media, use of serum concentrations lower than are generally used in cell culture, and the supplementation of trout embryo extract with growth promoting activity. ZEM cells are near-diploid and have been grown for approximately 40 population doublings. The ZEMH culture, initiated from haploid blastula-stage embryos, has been propagated for approximately 30 population doublings *in vitro* and exhibited a high proportion of haploid cells with the majority of the remaining cells exhibiting a hypoploid chromosome number.

The wide range in chromosome number in the ZEMH culture may be due to a gradual diploidization of the cell line as it is grown in culture. Spontaneous diploidization has been observed *in vitro* with haploid murine embryonal stem (ES) cells grown in culture and *in vivo* following transplantation of the haploid cells into a diploid embryo (Kaufman et al., 1983; Ishino et al., 1990). Alternatively, the range in chromosome number may reflect that the culture was derived from a pool of dissociated cells from several embryos possibly with different chromosome numbers. It should be possible to maintain a homogeneous population of haploid cells by routinely subcloning the cells or deriving cultures from individual haploid embryos.

All of the cultures required trout embryo extract and insulin in addition to FBS and trout serum during the initial passages in culture. Trout embryo extract promotes the serum-free growth of several established piscine cell lines, and its effect on the cells cannot be mimicked by purified mammalian growth factors (Collodi and Barnes, 1990). The partially purified mitogenic activity is heat stable and associates with two fractions of 25 and 60 kilo-daltons when analyzed by gel filtration chromatography or SDS polyacrylamide gel electrophoresis.

In most cases, cultures propagated for about 15 generations *in vitro* were able to grow in short-term experiments in medium supplemented with only FBS and trout serum. This change in phenotype may represent a metabolic adaptation of the cells to the culture conditions, or

may represent a selection during the culture period for a subpopulation of cells that were best suited to grow under the culture conditions. Such a subpopulation might be present initially in the cultures or might arise by genetic alteration upon proliferation *in vitro*.

In addition to the direct uses of zebrafish cell cultures in toxicology testing and mechanism studies and comparison with *in vivo* studies, zebrafish embryonal cell cultures represent a first step in developing the *in vitro* systems needed to conduct investigations of the genetic and biochemical parameters that influence embryonal cell growth and differentiation in this system. A potential application of this approach is the development of ES cell lines that can be utilized to produce chimeric embryos (Evans and Kaufman, 1981; Robertson et al., 1986).

The zebrafish blastula is comprised of indeterminant cells (Kimmel and Warga, 1986) that possess the potential to give rise to pluripotent cell lines, and when genetically manipulated, blastula-derived ES cells could be used as a vector to introduce foreign genes into the chimera, an alternative method of producing transgenic fish. This method provides an advantage over direct injection of DNA into the embryo because cells possessing the desired phenotype, such as the proper level of exogenous gene expression, can be selected *in vitro* before introduction into the embryo (Evans and Kaufman, 1981; Evans et al., 1983; Ishino et al., 1990; Nandi et al., 1988; Robertson et al., 1986; Collodi et al., 1992a).

Furthermore, the ability to grow fin cells from primary culture, demonstrated by the growth of the caudal and pelvic fins, enables one to screen for chimeras or carry out other genetic analyses using DNA derived from cultured cells initiated from fin tissue without sacrificing the embryo. Also, the ability to culture cells derived from liver, viscera, and gill makes possible an *in vitro* analysis of the tissue-specific expression of particular plasmid/promoter constructs used in producing the transgenic embryo and the tissue-specific expression of xenobiotic metabolizing enzymes (Helmrich et al., 1988; Bols and Lee, 1991; Pesonen et al., 1989; Pottenger and Jefcoate, 1990; Robertson et al., 1986; Stuart et al., 1988; Stuart et al., 1990; Collodi, et al., 1992a; Collodi et al., 1992b).

Another application of this approach to the production of transgenic animals is the use of homologous insertional mutation to produce ES cells deficient in specific gene products and thereby produce chimeric embryos exhibiting the same deficiency (Nandi et al., 1988). This application would be facilitated by the availability of a haploid ES cell line. We have demonstrated the feasibility of producing such a haploid cell line by initiating the ZEMH cell line from haploid embryos. ZEMH cells exhibit the ability to undergo differentiation in culture to a pigmented phenotype, making the cell line a potentially useful system for the *in vitro* study of cell differentiation.

The mammalian hypertetraploid cell line, C3H/10T1/2, developed from mouse embryos, has been shown to possess an intrinsic cytochrome P450 activity by its ability to metabolize polycyclic aromatic hydrocarbons (PAH) (Pottenger and Jefcoate, 1990). Pretreatment with

TCDD induces the PAH-metabolizing activity of these cells. In the present study, we demonstrate for the first time that fish cells (ZEMH), when exposed to TCDD in culture, were capable of expressing a new protein that was recognized by antitritout P450IA1 IgG in immunoblots. These TCDD-treated cells also displayed EROD activity which was not detected in the control cells. In an earlier study, TCDD was found to induce EROD activity in primary cultures of rainbow trout hepatocytes (Pesonen et al., 1989).

These findings suggest that TCDD induces the synthesis of a protein in ZEMH cells that is immunologically and functionally related to trout P450IA1. These zebrafish cultures may provide a unique model for toxicological studies involving the expression and regulation of PAH-metabolizing enzymes and their genes following exposure of the cells to environmental chemicals.

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Fish embryo cell cultures for derivation of stem cells and transgenic chimeras

Paul Collodi, Yuto Kamei, Angela Sharps,
Darin Weber, and David Barnes*

Department of Biochemistry and Biophysics,
Environmental Health Sciences Center, Oregon State
University, Corvallis, OR 97331, U.S.A.

Abstract

It has been demonstrated in mammalian systems that techniques using embryonal stem cells provide advantages over conventional injection of DNA into embryos for generation of transgenic animals. We employed cell culture approaches in an attempt to develop this technology for fish transgenesis. Using a trout embryo-derived mitogenic preparation in a specialized culture medium, we initiated replication of zebrafish blastula-derived cell cultures and expressed marker genes introduced into the cells by plasmid transfection. Reintroduction of cells from the cultures into blastula-stage embryos indicated that the cultured cells survived and may contribute to the developing organism.

Introduction

Introduction of foreign genes into developing embryos is a powerful approach to studying genes affecting cell differentiation and proliferation, as well as an instrumental method for more applied goals of biotechnology. Fish are becoming popular nonmammalian models of vertebrate development, but expression of integrated genes in transgenic fish is not easily obtained (Powers, 1989; Chen and Powers, 1990; Chen et al., 1990; Davies et al., 1990). A successful approach to this problem in mammals

uses embryonal stem (ES) cell cultures (Suemori et al., 1990; Soriano et al., 1986). Such embryo-derived cell lines grow indefinitely in vitro and also contribute to the developing organism (including germ line) when transplanted into the embryo. Thus, it is possible to transfect exogenous genes into ES cells, select in vitro, for the desired expression copy number and genomic organization, and then introduce the preselected cells into the embryo to create a chimeric transgenic animal (Gossler et al., 1986; Suemori et al., 1990; Wagner et al., 1985) (Figure 1). It is also possible to create organisms in which endogenous genes have been specifically altered using targeted gene inactivation of ES cells and by introducing ES cells into compromised embryos to create primary total transgenic-derived embryos (Robertson et al., 1986; Capecchi, 1990).

The ES cell approach has limited application in fish systems because of their lack of appropriate cell lines; we therefore initiated experiments to derive zebrafish (*Brachydanio rerio*) ES cell cultures. We applied approaches we used to derive diploid mouse embryo cell lines in serum-free or low-serum, hormone-supplemented media (Loo et al., 1987; Sakai et al., 1990). These cells do not exhibit growth crisis or chromosomal abnormalities and could not be derived in traditional, serum-supplemented culture medium because the cells are markedly growth-inhibited by the high concentrations of serum used in conventional culture. In applying this serum-free approach (Collodi et al., 1991a, 1991b) to fish embryo cell cultures, we found an essential mitogenic activity in extracts of trout embryos (Collodi and Barnes, 1990). We also adapted established approaches for plasmid transfection of mammalian cells to genetic manipulation of fish cell lines in vitro (Helmrich et al., 1988). We describe our progress in derivation of zebrafish cell cultures, transfection of fish cells, and injection of cultured cells into zebrafish embryos.

* Correspondence should be sent to this author at present address.

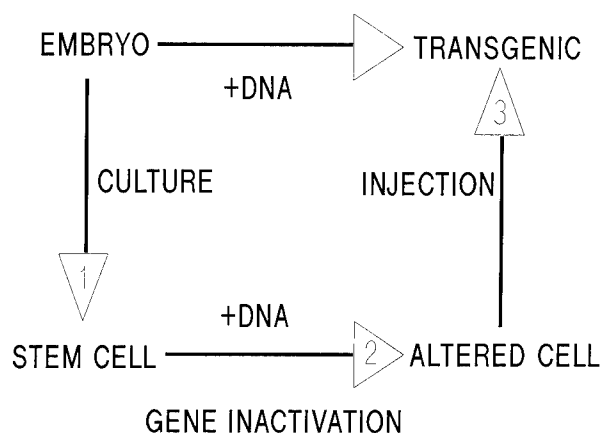


Figure 1. Production modes for transgenic animals. Conventional method for transgenic construction involves direct injection of plasmid DNA into a developing embryo (*top*). Derivation of transgenic chimeras with embryonic stem (ES) cells involves three steps: (1) culture of ES cells from embryos, (2) genetic manipulation *in vitro* to introduce exogenous genes or to inactivate endogenous genes and selection of the altered cells, and (3) reintroduction of altered cells into a developing embryo.

Results

Cell culture medium formulation

Mitogenic activity in extracts from 21-day-old trout embryos was necessary for serum-free growth of fish embryo cell lines. The embryo extract was mitogenically active at a protein concentration of 2 to 100 $\mu\text{g}/\text{mL}$ on a number of fish species (Figure 2) but did not stimulate DNA synthesis in quiescent mouse 3T3 fibroblasts over a range of concentrations (Collodi and Barnes, 1990). The mitogenic effect on fish cells could not be mimicked by purified mammalian growth factors.

Partial purification by hydrophobic interaction (phenyl-Sepharose) chromatography, ammonium sulfate precipitation, and preparative SDS-polyacrylamide gel electrophoresis identified peaks of activity with molecular weights of approximately 25,000 and 60,000. The partially purified material was active in the ng/mL range, completely destroyed as a result of incubation with trypsin, and stable to boiling and reduction. A low molecular weight and higher molecular weight activity peak were also obtained with Sephadex G100 gel filtration chromatography of the extract in 0.2 mol/L acetic acid, and preparative isoelectric focusing identified two peaks of activity (pI) (5.2 and 8.3). Activity was not retarded on heparin or concanavalin A affinity columns.

A complicated basal nutrient medium (LDF medium) was critical to the growth of zebrafish cells. The basal nutrient medium was supplemented with insulin (10 $\mu\text{g}/\text{mL}$), trout embryo extract (40 $\mu\text{g}/\text{mL}$), a small amount of trout serum (0.4%), and 0.1 mmol/L β -mercaptoethanol or 1% fetal bovine serum (FBS). β -Mercaptoethanol is commonly used in mouse ES cell cultures (Robertson, 1987). We also added conditioned medium (50%) from the BRL rat liver cell line cultured in serum-free LDF. This line has been used in culture of mammalian ES cells as a source of a factor that inhibits differentiation and maintains the ES cell phenotype (Smith and Hooper, 1987).

Cell culture derivation

Zebrafish embryo cultures were initiated from blastula, the developmental stage from which mammalian ES cells were derived (Robertson et al., 1986; Gossler et al., 1986; Robertson, 1987). We cultured zebrafish blastula-derived cells continuously for more than 40 population doublings (Figures 3, 4)

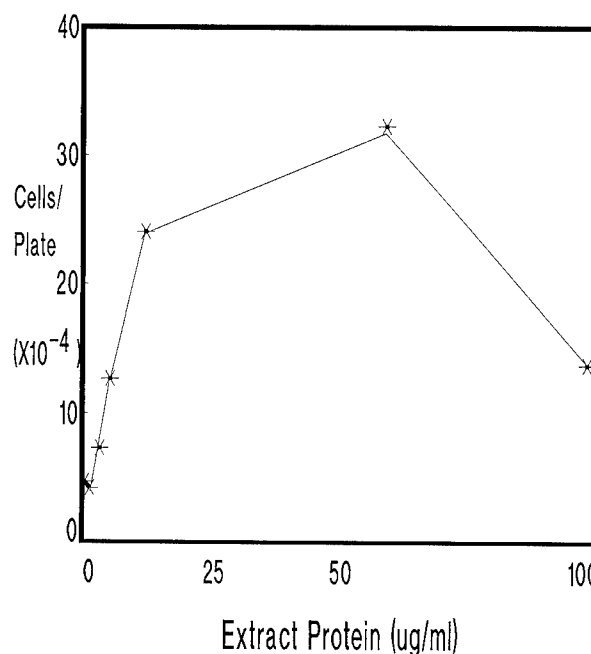


Figure 2. Stimulation of cell growth by partially purified trout embryo-derived mitogen. Salmon embryo (CHSE-214) cells were plated ($5 \times 10^4/35$ mm diameter plates) in LDF medium supplemented with bovine insulin (10 $\mu\text{g}/\text{mL}$), human transferrin (25 $\mu\text{g}/\text{mL}$), and the indicated concentration of partially purified trout embryo-derived mitogen. A suspension of trypsinized cells in PBS was counted 10 days after plating.

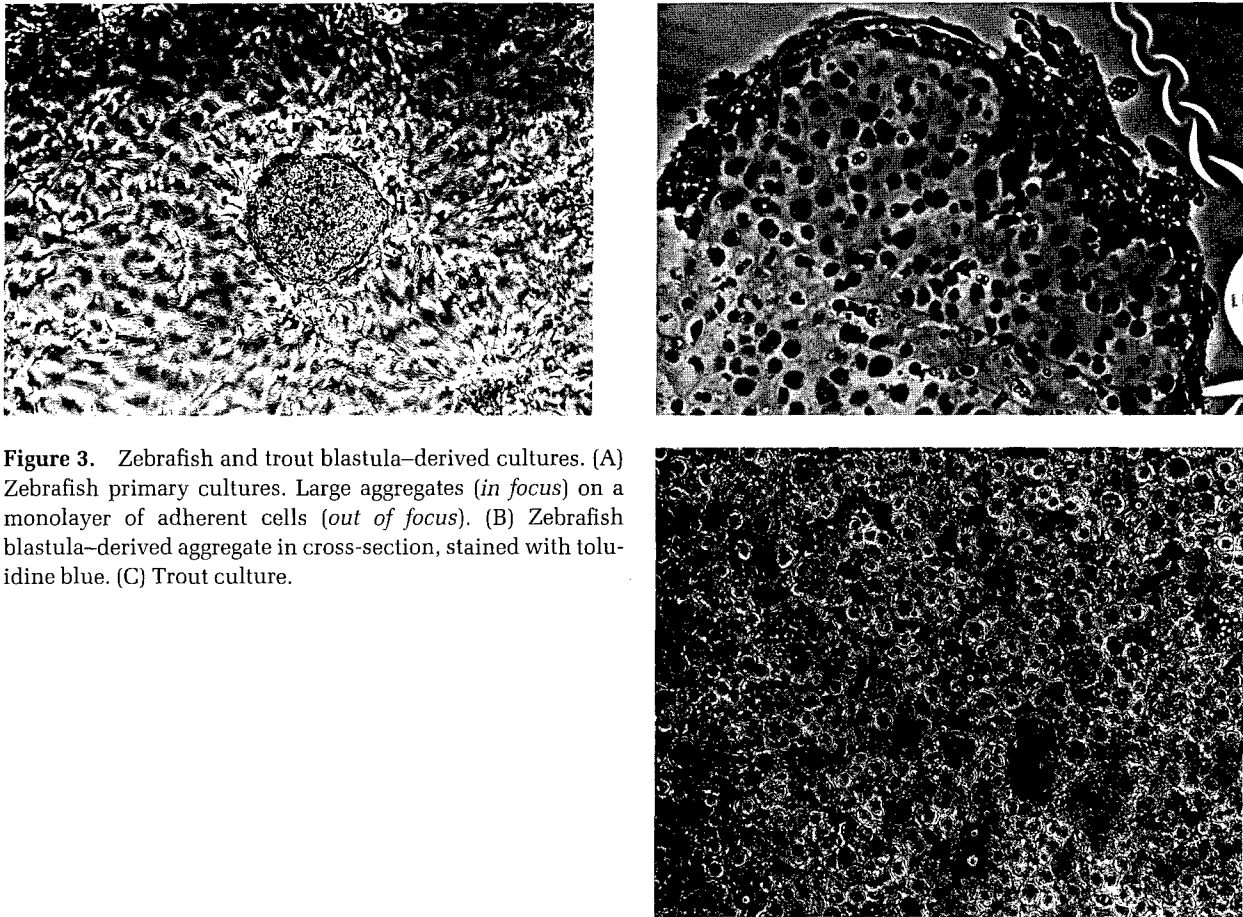


Figure 3. Zebrafish and trout blastula-derived cultures. (A) Zebrafish primary cultures. Large aggregates (*in focus*) on a monolayer of adherent cells (*out of focus*). (B) Zebrafish blastula-derived aggregate in cross-section, stained with toluidine blue. (C) Trout culture.

without a reduction in growth rate, and karyotyping showed that approximately 70% of the cells were still in the diploid range. Doubling time was approximately 72 hours. After approximately 2 weeks, primary zebrafish embryo cultures formed aggregates of cells growing on a layer of more strongly adherent cells, a phenomenon that is also observed with mouse blastula-derived cells (Robertson, 1987). Histological examination showed small cells within the aggregates that may have been undifferentiated ES cells, and a layer of larger, flattened cells outside the aggregate that may have been more differentiated than the inner cells (see Figure 3). Cultures were also initiated from blastula-stage (48 hr; 10°C) and later-stage (13 and 28 day old) trout embryos (see Figure 3). Trout blastula-derived cultures grew slowly with substantial cell death and were grown continuously for 50 population doublings in basal nutrient medium supplemented with insulin (10 µg/mL), trout embryo extract (20 µg/mL), and 1.0% FBS. Unlike zebrafish cultures, trout blastula-derived cultures generally did not adhere to the dish and grew as clumps or small groups of cells.

Extended culture of zebrafish blastula-derived cells in the absence of BRL-conditioned medium and without attempting to select for aggregates led to cultures consisting of primarily adherent cells (see Figure 4); a similar pattern is seen in the aggregates that develop in mouse blastula cultures (Robertson, 1987). Analogous approaches applied to adult zebrafish tissues allowed derivation of replicating cultures from fin, liver, viscera, and gill (see Figure 4) (Collodi et al., 1992). We also derived cell lines from blastula-stage haploid embryos produced by in vitro fertilization of zebrafish eggs with ultraviolet (UV)-inactivated sperm (see Figure 4). Cells of these cultures sometimes differentiated in vitro to a pigmented phenotype. Karyotype analysis of these cells after 30 population doublings in culture indicated that approximately 60% of the cells were haploid; flow cytometry also identified a population of cells in the haploid range (Figure 5). Both haploid- and diploid-derived cultures were from pooled embryos and have not yet been cloned to select for stable haploid or diploid karyotype.

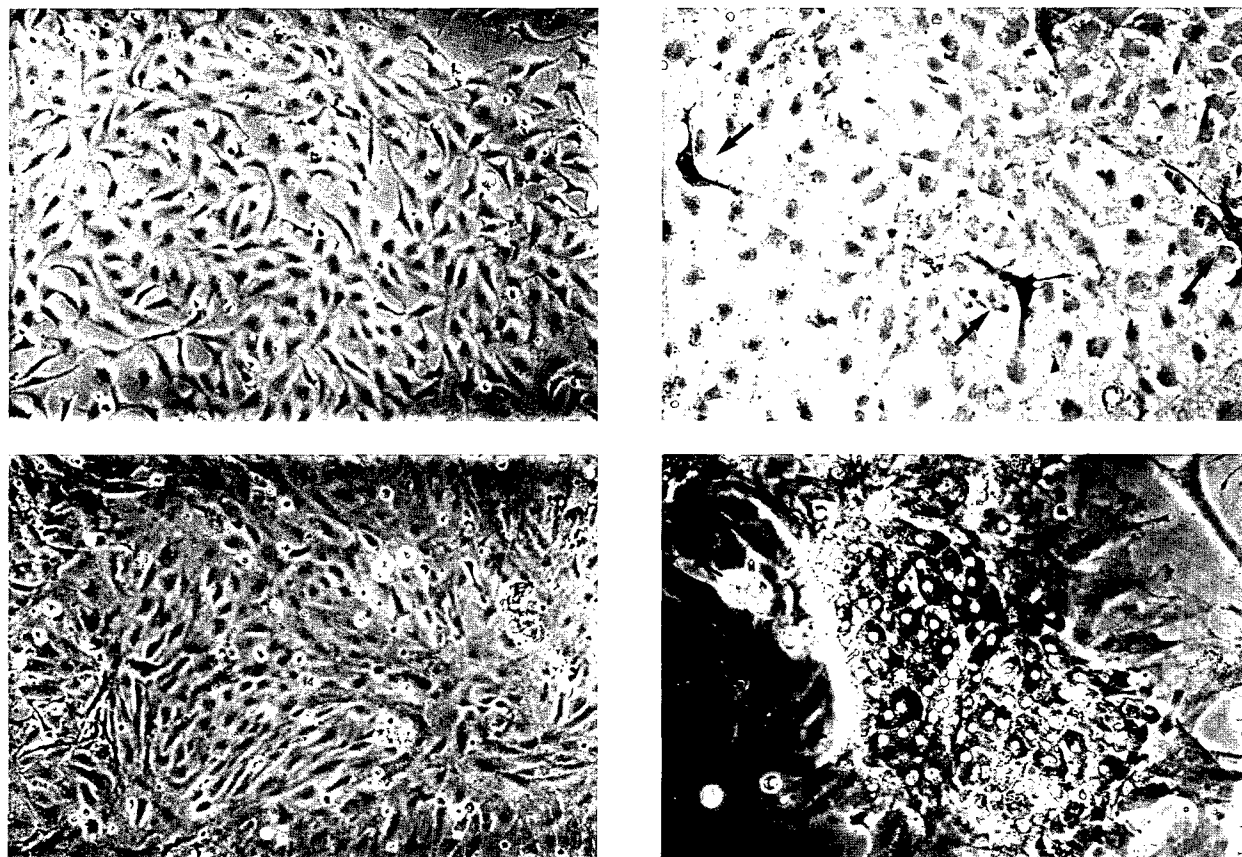


Figure 4. Zebrafish cell cultures on extended passage. (A) Diploid embryo-derived, approximately 40 population doublings; (B) haploid embryo-derived, approximately 35 population doublings, showing pigmented cells (arrows); (C) gill-derived, approximately 10 population doublings; and (D) liver-derived, approximately 5 population doublings.

Fish cell transfection and embryo injection

Although both trout and zebrafish represent interesting systems for generation of transgenic chimeras, we chose to concentrate on zebrafish because of the ease with which the cell cultures and fertilized zebrafish embryos can be manipulated. When cells from blastula-derived zebrafish cultures were labeled *in vitro* with rhodamine isothiocyanate (Snape et al., 1987) and introduced into blastulas, transplanted cells remained visible and viable in the embryo many hours after injection (Figure 6).

Successful generation of ES-derived transgenic zebrafish is also dependent on the ability to introduce exogenous DNA into the ES cell genome *in vitro*. We genetically marked zebrafish embryo cells by calcium phosphate-mediated transfection of the pSV2neo plasmid (Southern and Berg, 1982) containing the bacterial aminoglycoside phosphotransferase gene under control of the SV40 early promoter

with SV40 processing signals (Figure 7). We found that this plasmid conferred neomycin (G418) resistance in several fish cell lines through stable integration into the genome at frequencies comparable to those seen with mammalian cells (Helmrich et al., 1988). We injected neo-transfected zebrafish embryo-derived cells into blastulas and detected neo-specific DNA sequences in juvenile fish 18 days after injection (see Figure 7).

To explore the potential of other promoters and plasmid constructions, we compared transient expression of plasmids in which the bacterial β -galactosidase (β -gal) gene was expressed under control of the SV40 early promoter or cytomegalovirus (CMV) immediate early promoter and SV40 processing signals. Expression frequency in zebrafish cells was poor compared with carp (EPC cell line) and was similar to expression levels in salmon (CHSE cell line) (data not shown). Good expression with either

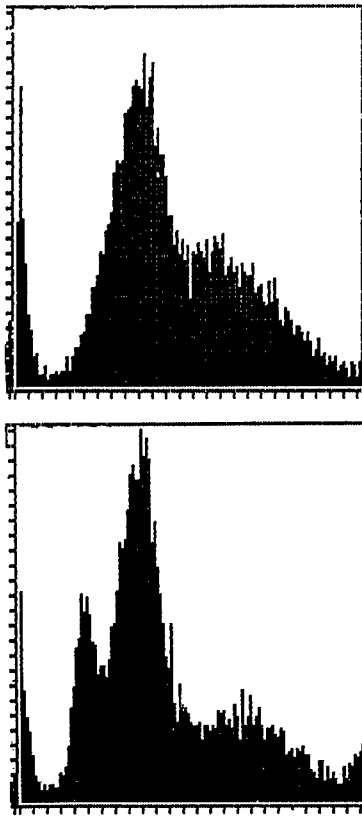


Figure 5. Flow cytometric analysis of diploid (*top*) and haploid (*bottom*) blastula-derived zebrafish embryo cells. Tracings show relative cell number (Y axis) versus relative amount of DNA per cell (X axis). Haploid-derived cultures show a haploid DNA content peak that is not present in the diploid-derived cultures.

promoter was dependent on the presence of a splice signal. To explore this phenomenon further, the CMV promoter/ β -gal plasmid construction without splice signal was introduced into cells in a coselection (*neo* gene with SV40 promoter also present on the plasmid), and G418-resistant colonies were selected. We observed expression of β -gal in only a small percentage of cells in the colonies (Figure 8), suggesting that low expression with this construction is the result of low expression in cells that contain the plasmid. A similar phenomenon is observed in mammalian cells with some plasmids (MacGregor et al., 1987).

Discussion

Virtually all developmental and transgenic studies with fish have employed *in vivo* approaches (Powers, 1989; Felsenfeld et al., 1990; Ho and Kane, 1990; Stuart et al., 1990; Chen and Powers, 1990), and practically no emphasis in these areas has been directed toward use of fish cell cultures. In addition to the practical potential of ES cell-based transgenics for deriving improved organisms, ES cells provide advantages for studying the role of specific gene products during development and exploring the biochemical and hormonal parameters that influence embryonal cell growth and differentiation.

Using the trout embryo-derived mitogen, we generated long-term, multipassage cultures from zebrafish fin, gill, gut, and liver, as well as from trout and zebrafish embryonic blastula. At this early

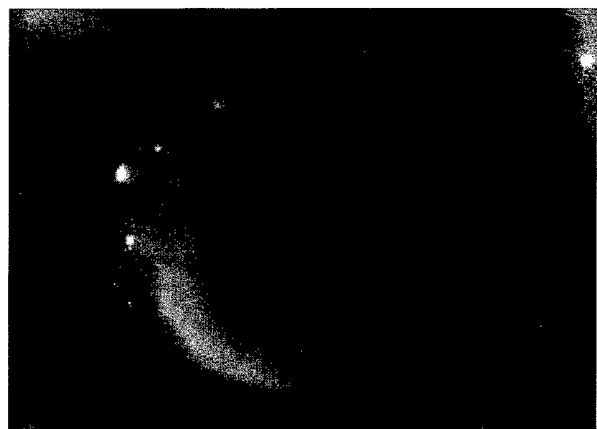
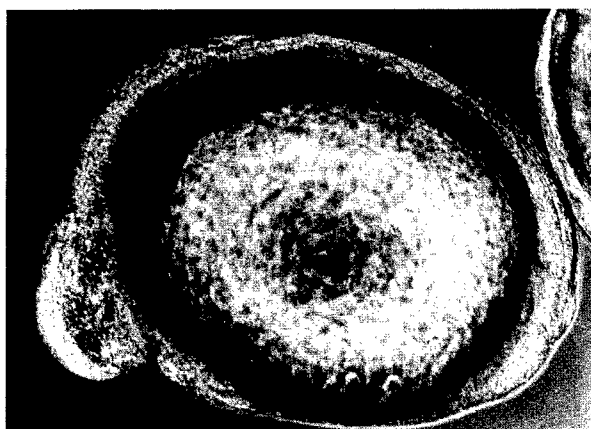


Figure 6. Zebrafish embryos injected with fluorescently labeled cells from blastula-derived cultures. Blastulas were injected with cultured cells labeled with tetramethyl rhodamine isothiocyanate, allowed to develop, and examined by phase (A) and fluorescence (B) microscopy 15 hours after injection. Cultured cells had undergone approximately 50 population doublings *in vitro* before reintroduction into the embryos.

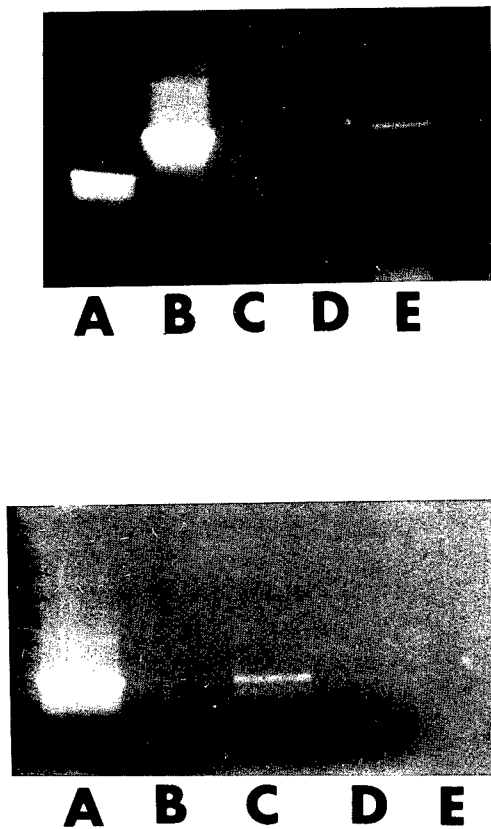


Figure 7. Transfected bacterial *neo* in cultured zebrafish embryo cells and zebrafish derived from transgenic chimeric blastulas. Shown are products of polymerase chain reaction (PCR), fractionated on 1% agarose gels and visualized by ethidium bromide staining. Upper panel PCR templates: lane A, 500-bp standard; lane B, pSV2*neo*; lane C, DNA from untransfected zebrafish embryo-derived cell culture; lane D, no DNA; lane E, DNA from pSV2*neo*-transfected, G418-selected zebrafish embryo-derived clone. Lower panel templates: lane A, pSV2*neo*; lane B, DNA from fish injected with control cells; lane C, DNA from fish 1 derived from blastula injected with *neo*-transfected cells; lane D, DNA from fish 2 derived from blastula injected with *neo*-transfected cells; lane E, blank. Primers for all lanes except lane A in upper panel were derived from *neo* to produce a 700-bp, PCR-amplified *neo* fragment. Primers for lane A in upper panel were provided by the manufacturer to amplify the 500-bp standard as a positive control. *Neo*-transfected cell clone used for blastula injections (lanes C and D, lower panel) was the clone represented by lane E in the upper panel. DNA was isolated from fish 18 days after cell injection.

developmental stage, zebrafish embryo cells are pluripotent (Hatta et al., 1991); they have the potential to give rise to ES cell cultures. ES cells cannot be maintained in conventional medium supplemented

only with serum as a source of growth factors; we use a complex formulation that includes BRL-conditioned medium. Work with mammalian ES cells has identified an interleukin-related differentiation/inhibitory activity produced by BRL cells that influences ES cells to maintain a pluripotent condition (Moreau et al., 1988).

A largely unexplored potential of ES cells is generation of haploid cultures. Fertilization of zebrafish eggs with UV-inactivated sperm produces haploid zebrafish embryos (Hatta et al., 1991) that develop well past the stages from which ES cell cultures are derived. Haploid blastula-derived clones specifically altered in a predetermined gene through targeted inactivation schemes and selections would provide useful lines for cell culture experiments. In addition, these cells could be diploidized by in vitro cell fusion techniques, the mutant homozygous diploid cells could be transplanted into embryos, and germ line transgenics could be identified to produce predetermined homozygous mutant organisms in a direct way.

We describe herein work regarding the three aspects of ES cell system development: (1) cell culture, (2) genetic alteration by transfection, and (3) reinjection of altered cells. We demonstrated long-term culture and transfection of zebrafish cells, although transfection frequency probably could be improved by altering vector constructions. Reinjection of fluorescently labeled and genetically marked cultured cells suggests that the cell culture system may be providing ES cells. We are currently testing the potential contribution of cells from our zebrafish embryo-derived cultures to tissues of the fully developed organism.

Experimental Procedures

Cell culture

Synchronously developing zebrafish blastula-stage embryos for initiation of cultures were obtained by in vitro fertilization. Fish were maintained on a 14-hour light/10-hour dark cycle; eggs and sperm were collected within 90 minutes after fish were exposed to light. Fish were anesthetized (3-amino-benzoic acid ethylester), and sperm from 2 or 3 males was collected in a capillary tube and stored on ice in a small volume of Hank's solution. Sperm remain viable for up to 90 minutes. Eggs were collected from a gravid female by gently pressing on the ventral side of the fish. Eggs were mixed with the sperm in a small petri dish, and fertilization was

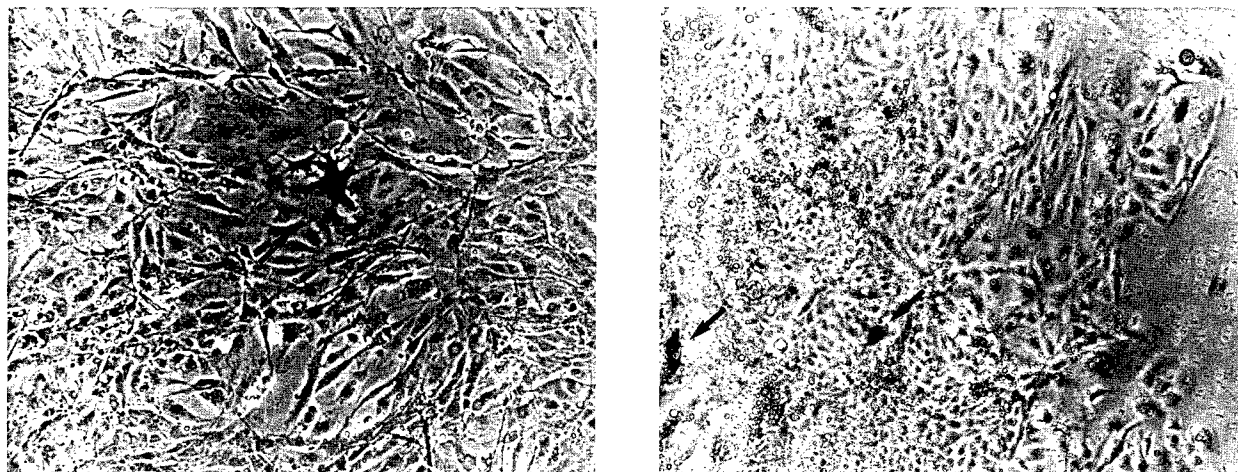


Figure 8. Expression of transfected β -gal in cultured fish cells. (A) Transient expression in zebrafish embryo culture 5 days after transfection (arrow shows cell expressing β -galactosidase). (B) Colony of G418-resistant salmon embryo (CHSE-214) cells selected after transfection of plasmid directing expression of both β -gal and *neo* (see text for plasmid construction). Only a few cells of the colony express β -galactosidase (arrows).

initiated by addition of 1 mL water to dilute the Hank's solution and to activate the sperm. After 2 minutes, additional water was added to the fertilized eggs, and embryos were allowed to develop undisturbed at 26°C. Blastula-stage embryos were harvested approximately 2 hours after fertilization.

To initiate cultures, individual embryos were confirmed microscopically to be at the correct developmental stage, rinsed in culture medium containing antibiotics (Collodi et al., 1991a, 1991b, 1992), soaked for 2 minutes in 0.5% bleach, and again rinsed. Individual or small groups of embryos were dechorionated and cells were disaggregated by trypsinization for 10 minutes and mechanical disruption by pipetting. The cell suspension was centrifuged, cells were resuspended in culture medium, and 0.1-mL aliquots of individual cells and small-cell aggregates were plated in 96-well tissue culture dishes. The following day, the medium was changed to remove lipid droplets and pieces of chorion. To generate haploid embryo-derived cultures, sperm was UV-irradiated (29 cm from a 43 cm Sylvania germicidal tube) for 2 minutes before fertilizing the eggs (Streisinger et al., 1981).

Cells were grown in LDF medium (50% Leibovitz's L-15 medium, 35% Dulbecco's modified Eagle's medium, and 15% Ham's F12 medium) supplemented with sodium bicarbonate (0.15 mg/mL), 15 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (pH, 7.2), penicillin (200 U/mL), streptomycin sulfate (200 μ g/mL), ampicillin (25 μ g/mL),

bovine insulin, trout embryo extract, trout serum, and FBS (Collodi et al., 1992c). BRL cultures were grown in serum-containing medium and changed to serum-free LDF medium when confluent for collection of conditioned medium. Conditioned medium was collected after 4 days, filtered, and used immediately or frozen in aliquots. BRL cells were maintained at 37°C, trout at 20°C, and zebrafish at 26°C. Trout embryo extract was prepared as described (Collodi and Barnes, 1990). Partial purification was achieved by phenyl-Sepharose chromatography (loaded in 2.5 mol/L NaCl/20 mmol/L PO_4 [pH, 7.2]; eluted in 20 mmol/L PO_4 /100 mmol/L NaCl [pH, 7.2]), followed by ammonium sulfate precipitation (50% saturated).

Karyotypic analysis

Flow cytometry was performed using an EPICS V flow cytometer. Cells were fixed and stained with chromomycin A3 as previously described (Rawson et al., 1991). Karyotyping was performed as previously described, with modifications developed for fish cultures (Loo et al., 1989; Collodi et al., 1992). Rapidly growing cultures were incubated for 9 hours in colcemid (0.2 μ g/mL). Cells were trypsinized and centrifuged; the pellet was gently resuspended in 0.4% KCl and incubated at room temperature for 20 minutes, followed by fixation and washes in methanol/acetic acid (3:1). Chromosome spreads were prepared and stained in 3% Giemsa solution.

Embryo injections

Cells were labeled in vitro with tetramethyl rhodamine isothiocyanate as previously described (Snape et al., 1987), harvested by trypsinization, washed, and suspended (5×10^6 /ml) in LDF medium with 1% phenol red to visualize the solution during injection. Synchronously developing embryos from in vitro fertilization were dechorionated in pronase (0.5 mg/mL) and transferred to a small dish containing LDF medium. Several microliters of cell suspension were loaded into a glass micropipet prepared from a 1.2-mm fiber-filled glass capillary tube drawn to a 20 micrometer/L diameter tip, and the micropipet was loaded into a pneumatic microinjector with a micromanipulator. Using an inverted phase microscope, the micropipet was positioned above the dechorionated embryo and inserted into the center of the cell mass; a small volume (1–2 nL) of cell suspension was released into the embryo. Survival was approximately 30%, and approximately 50 embryos could be injected in 1 hour. After 48 hours the embryos were transferred to distilled water containing 60 mg/L Instant Ocean. Embryos were injected with approximately 10 cells.

Plasmid transfection and expression

We obtained a plasmid containing β -gal with CMV immediate early promoter, SV40, small splice site, and polyadenylation signal (MacGregor and Caskey, 1989) from Dr D. Grunwald, University of Utah. A similar construction (without the splice site) was obtained from Dr S. Shirahata, Kyushu University. This plasmid also contained the bacterial aminoglycoside phosphotransferase (*neo*) gene under control of SV40 regulatory sequences. A plasmid containing β -gal with SV40 early promoter, small t-splice site, and polyadenylation signal was obtained from Dr G. Merrill, Oregon State University, and a similar plasmid without the splice site was constructed in our laboratory. The pSV2*neo* plasmid was obtained from the American Type Culture Collection.

Transfections and selections for G418-resistant fish cells were performed as previously described (Helmrich et al., 1988). Expression of β -gal was evaluated by colorimetric enzyme assay (Suemori et al., 1990). Lightly fixed cells (2% formaldehyde/0.2% glutaraldehyde, 5 minutes, 4°C) were incubated in 5-bromo-4-chloro-3-indolyl- β -D-galactoside with K ferricyanide/ferrocyanide and detergent at 37°C for 24 to 48 hours. A blue color develops if the enzyme is expressed.

Polymerase chain reaction (PCR) was carried out using a commercial kit (GeneAmp; Perkin Elmer

Cetus) as recommended by the vendor in an Ericomp twinblock thermal cycler (one cycle: 94°C/4 min, 55°C/1 min, 72°C/2 min; 40–50 cycles: 94°C/1 min, 55°C/1 min, 72°C/2 min). For preparation of DNA templates from cultured cells (Shalnik and Orkin, 1990), flasks were trypsinized, and the cell pellet was digested with proteinase K (20 μ g/mL, 30 min, 55°C) in 50 mmol/L TRIS (pH, 8.0), 20 mmol/L NaCl, 1 mmol/L EDTA, and 1% SDS. After digestion, samples were boiled and used directly for PCR. Washed embryos were similarly treated for preparation of DNA templates from fish.

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Induction of zebrafish (*Brachydanio rerio*) P450 *in vivo* and in cell culture

P. COLLODI†*, C. L. MIRANDA‡§, X. ZHAO‡, D. R. BUHLER‡§ and D. W. BARNES†

† Department of Biochemistry and Biophysics,

‡ Department of Agricultural Chemistry, and

§ Marine/Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, OR, USA

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1. Induction of zebrafish P450 by 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) was studied in liver tissue, primary liver cell culture and multipassage cell culture derived from zebrafish haploid and diploid embryos and liver.

2. TCDD induced two hepatic proteins (54 and 50 kDa) *in vivo* which were recognized by anti-trout P4501A1 IgG. The 54-kDa protein was induced by TCDD in primary and multipassage hepatocyte cultures and in haploid and diploid embryo-derived cells. The proteins in liver homogenates were not induced by aqueous exposure of zebrafish to β -naphthoflavone (BNF).

3. Homogenates of zebrafish liver, cultured hepatocytes and embryo-derived cells also exhibited increased ethoxyresorufin O-deethylase (EROD) and 7-12-dimethylbenz[*a*]anthracene (DMBA) hydroxylase activity following TCDD exposure.

Introduction

The fish is a popular experimental organism for studies of P450 regulation (Stegeman and Lech 1991, Andersson and Forlin 1992). As with other vertebrates, the fish P450-dependent, mixed-function oxidase system plays a critical role in the metabolism of xenobiotics (Lorenzen and Okey 1990, Pesonen and Andersson 1991, Pesonen *et al.* 1992). Salmonids, such as the rainbow trout, have often been used for these studies. However, recent attention has focused on the use of small aquarium species such as the medaka and zebrafish which grow rapidly, reach sexual maturity in about 3 months, and provide large numbers of synchronously developing embryos year round (Aoki and Matsudaira 1977, Egami *et al.* 1981, Hatanaka *et al.* 1982, Bresch *et al.* 1990, Babich and Borenfreund 1991, Bresch 1991). *In vivo* studies concerning the effects of xenobiotic exposure at various stages of the zebrafish life cycle have been informative (Bresch *et al.* 1990, Bresch 1991, Dave and Xiu 1991, Nagel *et al.* 1991). However, *in vitro* approaches utilizing cells grown in culture have not been applied to the zebrafish due to the lack of suitable cell culture systems.

In other species such as salmonids, work with cultured cells has complemented whole animal studies of toxicology (Smolarek *et al.* 1987, Pesonen and Andersson 1991, Pesonen *et al.* 1992). Cultured cells have been used to study the extracellular factors which induce cytochrome P450. Polycyclic aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and β -naphthoflavone (BNF) have been shown to be potent inducers of P4501A1 in primary rainbow trout hepatocyte cultures (Pesonen and Andersson 1991, Pesonen *et al.* 1992). TCDD is also an

*Present address and author for correspondence: Department of Animal Sciences, Purdue University, West Lafayette, IN 47907, USA.

inducer of aryl hydrocarbon hydroxylase (AHH) activity in the rainbow trout hepatoma cell line RTH-149 (Lorenzen and Okey 1990).

Characterization of P450 regulation and development of cell culture systems are important in order to fully utilize the zebrafish as a model for toxicology research. Recently, we have developed methods to derive long-term cell cultures from blastula-stage diploid and haploid zebrafish embryos and from several adult tissues including fin, gill, viscera and liver (Collodi *et al.* 1992a). Upon exposure to TCDD the embryo-derived cells expressed an inducible protein that was immunologically and functionally similar to rainbow trout P4501A1 (Collodi *et al.* 1992a, b). Here we compared the induction of zebrafish P450 *in vivo* and in primary hepatocyte cultures and multipassage cultures of cells derived from liver and haploid and diploid embryos of zebrafish.

Materials and methods

Chemicals

Culture media (Leibovitz's L-15, Dulbecco's modified Eagle's and Ham's F12 media), foetal bovine serum and insulin were purchased from Gibco BRL (Gaithersburg, MD, USA). Mouse epidermal growth factor (EGF) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Resorufin, BNF and 7,12-dimethylbenz[a]anthracene (DMBA) were purchased from Sigma (St Louis, MO, USA). 7-Ethoxyresorufin was purchased from Molecular Probes, Inc. (Eugene, OR, USA). 7-12 [G - 3H]dimethylbenz[a]anthracene was purchased from NCI Radiochemical Repository, Chemsyn Science Laboratories (Lenexa, KA, USA). TCDD was purchased from ANALABS (New Haven, CT, USA).

Cell culture

Adult zebrafish were killed in iced-water, sterilized with 70% (v/v) ethanol and rinsed in sterile phosphate-buffered saline (PBS) (13 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 135 mM NaCl, 2.5 mM KCl; pH 7.0). Livers dissected from 10 fish were combined and incubated for 5 min at room temperature in 3 ml trypsin (0.2% (w/v) trypsin, 1 mM EDTA in PBS; pH 7.2). The tissue was then gently dissociated by pipetting to form a suspension of individual cells and small cell aggregates. The cells were collected by centrifugation (500g), washed once with LDF culture medium (50% (v/v) Leibovitz's L-15, 35% (v/v) Dulbecco's modified Eagle's and 15% (v/v) Ham's F12 media) supplemented with sodium bicarbonate (0.15 mg/ml), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (hepes) buffer (pH 7.2), penicillin (200 IU/ml), streptomycin sulphate (200 μ g/ml), ampicillin (25 μ g/ml) (Collodi *et al.* 1992a) and finally resuspended in 6 ml LDF. The cell suspension was then added to a six-well tissue culture plate (Falcon) (1 ml/well) and the plate allowed to sit undisturbed at room temperature for 30 min to enable individual cells and cell aggregates to attach to the plastic. An additional 1 ml LDF was gently added to the dish along with the following supplements: 5% (v/v) foetal bovine serum, 10 μ g/ml bovine insulin, 0.5% (v/v) trout serum, 20 ng/ml EGF and 40 μ g/ml trout embryo extract. Trout serum and embryo extract were prepared as previously described (Collodi and Barnes 1990, Collodi *et al.* 1992a). Individual dishes were wrapped in parafilm to prevent drying and incubated at 26°C. After about 7 days the cells grew to confluence and were used for the exposures. Multipassage cultures of liver (Miranda *et al.* 1993) and embryo-derived cells (Collodi *et al.* 1992a) were initiated and maintained as described. Liver cell cultures derived under these conditions exhibit characteristics common to parenchymal cells including glucose 6-phosphatase enzyme activity and serum albumin synthesis (Ghosh *et al.* 1994).

Cell exposures and subcellular fractionation

Confluent liver cell primary cultures grown in six-well tissue culture dishes (about 5×10^5 cells/well) and multipassage cultures of zebrafish liver and haploid and diploid embryo-derived cells were exposed to 10 nM TCDD or 0.1% (v/v) dimethylsulphoxide (DMSO) (vehicle control). After 48 h the cells were washed twice with phosphate-buffered saline (PBS) and scraped from the plate in 1 ml buffer A (50 mM Tris acetate buffer, pH 7.5, containing 20% glycerol and 0.1 mM EDTA). The cells were collected by centrifugation, resuspended in buffer A and homogenized using a Dounce homogenizer. For subcellular fractionation, the cell pellets were homogenized in 50 mM Tris acetate buffer, pH 7.5, containing 0.1 M potassium chloride, 1 mM EDTA and 0.1 mM phenylmethylsulphonylfluoride (PMSF). The homogenates were centrifuged at 8700g for 15 min. The supernatant was then centrifuged at 105 000g for 90 min. The 8700g pellet (nuclei and mitochondria) and 105 000g pellet (microsomes) were rehomogenized in buffer A prior to storage at $-80^\circ C$.

In vivo exposures

Adult zebrafish were treated in static water for 48 h with DMSO, 2 µl/l; BNF, 50 µg/l; or TCDD, 0.4 µg/l. At the end of the exposure period the fish were killed and the livers removed and homogenized in buffer A.

Measurement of catalytic activities

Ethoxyresorufin *O*-deethylase (EROD) activity of the liver cells was determined by the method of Prough *et al.* (1978). DMBA hydroxylase activity was determined using [³H]-DMBA as substrate by the method of De Pierre *et al.* (1978). The metabolites of [³H]-DMBA were analysed by hplc (Smolarek *et al.* 1987) using a Zorbax ODS column (4.6 × 250 mm) connected to a Radiomatic detector. Protein content was determined by Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard and appropriate dilutions of buffer A as reagent blanks to correct for the absorbance given by Tris.

Immunoblotting

Immunoblot (Western) analysis was used to detect the presence of P450 forms in the liver tissue and cultured cells that are homologous to rainbow trout P4501A1 (Miranda *et al.* 1989). The protein samples, separated by electrophoresis in 8% (w/v) SDS-polyacrylamide gels, were electrotransferred to nitrocellulose (Burnette 1981). After blocking with 2% (w/v) BSA, the nitrocellulose sheets were exposed to anti-rainbow trout P4501A1 IgG (Williams and Buhler 1984) and then to [¹²⁵I]-protein A for detection of immunoreactive bands on Kodak XAR film.

Results

Liver homogenates prepared from TCDD-treated zebrafish contained a 54-kDa protein recognized by anti-rainbow trout P4501A1 IgG (figure 1, lanes 7 and 8). This form differed in apparent molecular weight from the rainbow trout P4501A1 isoform (mw = 58 000; lanes 4 and 5). The homogenates also contained a 50-kDa protein which reacted less strongly with the antibody or was present at a lower concentration. Neither protein was found in liver homogenates prepared from BNF-treated or non-treated control fish (figure 1, lanes 1–3 and 6).

Two immunoreactive bands were also detected in homogenates prepared from TCDD-treated primary (figure 2, lane 1) and multipassage (figure 2, lane 3) zebrafish liver cell cultures. The proteins persisted at the induced level for a minimum of 7 days following the removal of TCDD from the multipassage liver cell cultures (figure 2, lane 4). The 54-kDa protein was more abundant than the 50-kDa protein in homogenates prepared from the cultured cells.

The subcellular distribution of the 54-kDa protein induced by TCDD in primary hepatocyte cultures is shown in figure 3. The 54-kDa protein recognized by anti-trout P4501A1 IgG was present in the 105 000g pellet (microsomes) (lane 7) and in the 8700g pellet (nuclei and mitochondria) (lane 4) from TCDD-treated cultures. The presence of the induced protein in the 8700g pellet may be due to microsomal contamination. No immunoreactive 54-kDa protein was detected in subcellular fractions from DMSO-treated cells (lanes 1, 3 and 6). A 50-kDa protein was also present as a very faint band in the 8700g pellet (nuclei and mitochondria) but was not detected in the microsomal fraction.

TCDD exposure greatly induced EROD activity in the zebrafish liver homogenates and in the primary hepatocyte cultures (table 1). EROD activity of liver homogenates increased nine-fold after the zebrafish were exposed to TCDD (0.4 µg/l) in static water for 48 h. Addition of 10 nM TCDD to primary hepatocyte cultures resulted in an increase in EROD activity from a non-detectable level to 62.5 pmol/min/mg protein in the microsomal fraction (105 000g pellet). EROD activity was also present in the nuclear plus mitochondrial fraction (8700g pellet), however no attempt was made to wash the 8700g pellet before resuspending in buffer A, therefore this activity may be partially due to microsomal contamination. TCDD treatment also induced DMBA hydroxylase activity in liver homogenates and in the

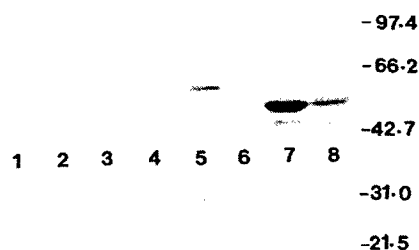


Figure 1. Immunoblot of liver homogenates from DMSO-, BNF- and TCDD-treated zebrafish probed with anti-trout P4501A1 IgG. Adult zebrafish were treated in static water for 48 h with DMSO, 2 μ l/l; BNF, 50 μ g/l; or TCDD, 1 nM. Liver homogenates were prepared as described in the Materials and methods. Samples (60 μ g protein/lane) were applied as follows: lanes 1 and 2, DMSO; 3 and 6, BNF; 7 and 8, TCDD; 4, purified trout P4501A1 (0.25 pmol); and 5, purified trout P4501A1 (0.50 pmol). Duplicate lanes show samples prepared from different fish.

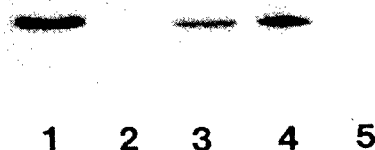


Figure 2. Immunoblot of homogenates (20 μ g/lane) from zebrafish primary (lanes 1 and 5) and multi-passage (3 and 4) hepatocyte cultures probed with anti-trout P4501A1 IgG. Cells were treated and homogenates prepared as described in the Materials and methods. Cells were treated with DMSO (lane 5) or TCDD (1 and 3) for 48 h before analysis. In lane 4 cells were treated with TCDD (10 nM) on days 1 and 2, refed with fresh medium (no TCDD) on day 3 and harvested on day 10. Purified trout P450 (0.063 pmol) was applied on lane 2 as a standard.



Figure 3. Immunoblot of subcellular fractions from zebrafish primary hepatocyte cultures treated with DMSO (control) or TCDD (10 nM). The blot was probed with anti-trout P4501A1 IgG. Samples (20 μ g/lane) were applied as follows: Lane 1, control homogenate; 2, homogenate from TCDD-treated hepatocytes; 3, control nuclei plus mitochondria; 4, nuclei plus mitochondria from TCDD-treated hepatocytes; 5, purified rainbow trout P4501A1 (0.5 pmol); 6, control microsomes; and 7, microsomes from TCDD-treated hepatocytes.

Table 1. Effect of TCDD treatment on EROD and DMBA-OH activity in zebrafish liver homogenates†, primary hepatocyte cultures‡ and cultured cells derived from zebrafish haploid and diploid embryos‡.

Subcellular fraction	EROD activity*		DMBA-OH activity*	
	DMSO	TCDD	DMSO	TCDD
<i>In vivo</i> exposure				
Liver homogenate	6.0	55.0	1.3	35.2
Hepatocyte cultures				
Homogenate	3.0	65.0	5.0	102.0
Nuclei and mitochondria	0	78.9	18.6	101.0
Microsomes	0	62.5	0.0	54.0
Haploid cell				
Microsomes	0	11.6	8.6	27.0
Diploid cell				
Microsomes	0	6.5	0.3	13.6

† Adult zebrafish were exposed to TCDD in static water (1 nM for 48 h) before killing. Values represent means from two measurements with pooled samples from two livers.

‡ Cells were exposed to TCDD (10 nM) or DMSO (0.1%, v/v) for 48 h before being harvested.

* Activity is expressed as pmol/min/mg/protein.

cultured hepatocytes (table 1). Elevated levels of DMBA hydroxylase activity were detected in all three subcellular fractions of the primary cultures.

The EROD and DMBA hydroxylase activities of multipassage cell cultures derived from haploid and diploid zebrafish embryos are also presented in table 1. EROD activity was not detected in DMSO-treated embryo cells, but upon exposure to TCDD the cells displayed EROD activity which was 10–20% of that exhibited by microsomes prepared from the TCDD-treated primary hepatocyte cultures. Microsomal DMBA hydroxylase activity was increased three-fold in haploid embryo-derived cells and 45-fold in diploid embryo-derived cells after TCDD treatment. However, the induced DMBA hydroxylase activity of the embryo-derived cells was still lower than that of the primary hepatocyte cultures.

Discussion

The results demonstrate that *in vivo* exposure of zebrafish to TCDD results in the induction of two hepatic proteins (54 and 50 kDa) which are immunologically related to trout P4501A1. The proteins were not detected in liver homogenates of BNF-treated fish. The same pattern of induction was exhibited *in vitro* in primary and multipassage zebrafish hepatocyte cultures treated with TCDD. Previously, we have shown that the 54-kDa protein is also induced in TCDD-treated zebrafish embryo cell cultures (Collodi *et al.* 1992b) and long-term cultures of zebrafish liver cells exposed to different concentrations of TCDD (Miranda *et al.* 1993).

In other fish species, inducible hepatic proteins recognized by anti-P4501A1 IgG vary in apparent molecular weight from 54 to 59 kDa (Goksoyr *et al.* 1991). However, in each species only one protein is recognized by the antibody. Detection of both the 54 and 50 kDa proteins in zebrafish liver may indicate that two forms of P450 are induced by TCDD. However, it is possible that the 50-kDa protein could be a degradation product of the 54-kDa protein. Purification and further study of each protein is needed to determine whether both of these proteins represent inducible forms of zebrafish P450.

BNF treatment did not induce the zebrafish P4501A1-related proteins in liver homogenates. This finding is consistent with the lack of an inducing effect of BNF in multipassage cultures of zebrafish liver cells (Miranda *et al.* 1993). The concentration of 50 μ g BNF/l used in the *in vivo* zebrafish exposure was effective in inducing P4501A1 protein and mRNA in rainbow trout (Haasch *et al.* 1993). These results indicate that induction of the zebrafish P4501A1 system is more selective than in other fish species. More research is required to determine if intraperitoneal or dietary administration of BNF is ineffective in inducing P4501A1-like proteins in zebrafish. Further studies are also needed to determine if the induction exhibited by zebrafish hepatocytes is receptor mediated and if the increased specificity is due to a more restrictive ligand specificity of the zebrafish Ah receptor.

In rainbow trout, hepatic microsomal EROD (Andersson and Forlin 1992) and DMBA hydroxylase (Henderson *et al.* 1992) activities are induced by BNF, a known inducer of trout P4501A1. The TCDD-induced increases of these enzyme activities in zebrafish liver homogenates, in primary hepatocyte cultures and multipassage cultures of haploid and diploid embryo-derived cells in culture provide further evidence of a P4501A type induction by TCDD in zebrafish cells *in vivo* and *in vitro*.

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